Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 1, 6-11, 18-28 are pending in the application, with 1 and 28 being the independent claims. Claim 1 has been amended to clarify that the method does not include raising the temperature of the mixture above the cloud point at any point during the process. Support for the amendment can be found in the specification at paragraph [0067].

The Examiner has indicated that claims 23-27 are allowable. (*See* Office Action, hereinafter "OA," at page 6.)

New claims 28-42 has been added, support for the amendment can be found in original claims 1 and 23-27. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Rejections under 35 U.S.C. § 103

Claims 1-8 and 10-22

The Examiner has rejected claims 1-8 and 10-22 under 35 USC § 103(a) as allegedly unpatentable over International Published Application No. WO 02/00844 ("Evans") in view of U.S. Patent No. 5,811,088 ("Hunter"). (OA at page 2.) The Examiner alleges that Evans teaches formulating DNA vaccines by mixing cationic surfactant BAK, a POP-POE copolymer (such as CRL 1005) and a polynucleotide at a

temperature below the cloud point of the copolymer (2-7°C). (OA at page 2.) "Evans stated that the inclusion of the cationic surfactant results in an increased percentage of polynucleotide that is physically associated with the block copolymer/cationic surfactant upon mixing and/or temperature cycling through the block copolymer cloud point. . . " (OA at page 3) (internal citation omitted.) Based on this single paragraph in Evans, the Examiner asserts "[t]hus Evans does not require "temperature cycling" through the cloud point" (OA at page 3 and 6.) The Examiner asserts that "[o]ne of ordinary skill in the art at the time of the invention would appreciate that Evans performed temperature cycling in order to adjust the size of particles formed by mixing the cationic surfactant, the POP-POE copolymer and the polynucleotide." (OA at page 3.) The Examiner alleges, "[h]owever, it is clear from the disclosure of Evans that the formation of the particle is what is desired, and that temperature cycling is not necessary to obtain it." (OA at page 3.) The Examiner acknowledges that Evans did not teach a cold filtration step, this step is allegedly taught by Hunter. The Examiner asserts that adding the cold filtration step would be obvious because the composition is intended to be used as a vaccine. Applicants respectfully traverse this rejection as it may apply to the currently pending claims.

The factors to be considered under 35 U.S.C. § 103(a), are the scope and content of the prior art; the differences between the prior art and the claims at issue; and the level of ordinary skill in the pertinent art. *See Graham v. John Deere*, 86 S.Ct. 684 (1966) and MPEP §2141. This analysis has been the standard for 40 years, and remains the law today. *See KSR International Co v. Teleflex Inc.*, 127 S.Ct. 1727 (2007).

A *prima facie* case of obviousness may be rebutted by a showing that the references or art at the time the invention was made teaches away from making a particular combination. "The court relied on the corollary principle that when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious." *See KSR International Co v. Teleflex Inc.*, 127 S.Ct. 1727, at 1740 (2007), citing *United States v. Adams* 383 U.S. 39, at 51-52.

A prior art reference must be considered in its entirety, *i.e.*, as a whole, including portions that would lead away from the claimed invention. Distilling an invention down to the "gist" or "thrust" of an invention disregards the requirement of analyzing the subject matter "as a whole." *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984).

Additionally, objective evidence or secondary considerations such as unexpected results, commercial success, long-felt need, failure of others, copying by others, licensing, and skepticism of experts are relevant to the issue of obviousness and must be considered in every case in which they are present. *See* MPEP §2141. Here, even if the references could be properly combined under Graham (which they cannot) and disclosed all of the elements of the presently claimed invention (which they do not), the present invention provides at least one secondary indicia of nonobviousness over such a theoretical combined disclosure of the cited art - unexpected results. This has long been recognized as classical secondary indicia of nonobviousness, and is evident in the present case. *See Graham v. John Deere Co.*, 86 S.Ct. 684, 694 (1966); *Custom Accessories v.*

Jeffrey-Allan Industries, 807 F.2d 955, 960 (Fed. Cir. 1986); *In re Soni*, 54 F.3d 746, 750 (Fed. Cir. 1995).

The presently claimed methods are directed to mixing a cationic surfactant, a polyoxyethylene (POE) and polyoxypropylene (POP) block copolymer, and a polynucleotide at a temperature below the cloud point of said block copolymer to form a mixture, and cold filtering the mixture to produce a sterile formulation. At no point in this process is the temperature raised above the cloud point. Examples 2 and 8 of the present application describe the production of sterile formulations using the method of claim 1. The cited references do not disclose a process of cold filtering a mixture of a cationic surfactant, copolymer and polynucleotide where the temperature is maintained below the cloud point of the copolymer throughout the production process. The cited reference teaches away from filtering a mixture comprising a polynucleotide and cationic surfactant. The discovery that the mixture of a cationic surfactant, copolymer and polynucleotide can be filtered is unexpected.

Maintaining the temperature below the cloud point

Contrary to the Examiner's assertion that "Evans does not require "temperature cycling" through the cloud point" (OA at page 6), all of the examples in Evans require that the temperature of the mixture is raised through the cloud point at least once. Evans page 3, line 6-11, does not support the Examiner's assertion that the mixture is not raised through the cloud point at least once. Temperature cycling is the process of repeatedly bringing the temperature of the mixture above the cloud point, followed by reducing temperature below the cloud point and then raising the temperature again. Evans requires that the block copolymer, cationic surfactant and polynucleotide mixture be

raised above the cloud point at least once. The language in the cited section is: mixing and/or temperature cycling through the cloud point. (*See* page 3, line 6-11) This can be interpreted as (a) mixing through the cloud point; or (b) temperature cycling through the cloud point; or (c) mixing and temperature cycling through the cloud point. Any one of the interpretation (a)-(c) requires that the temperature is raised through the cloud point at least once. Here, the claims do not require that the temperature is raised above the cloud point at any time during the mixing and filtration process. Thus, the present invention differs from the cited reference. Evans lacks the element of maintaining the temperature below the cloud point at all times during the production procedure.

Evans does not teach a method of producing a cationic surfactant, block copolymer and polynucleotide formulation without raising the temperature at least once in the production process. (*See* page 3, lines 6-11, paragraph spanning pages 32-33.) The Examiner asserts "that the formation of the particle is what was desired, and that temperature cycling was not necessary to obtain it." (OA at page 3.) A reference needs to be considered "as a whole" and cannot be distilled down to its gist as suggested by the Examiner. (*See W.L. Gore & Associates, Inc. v. Garlock, Inc.* 721 F.2d 1540 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984).) All formulations described by Evans were prepared by mixing the pure copolymer with cold plasmid DNA. BAK is then added to the cold DNA/copolymer mix. "After the addition of BAK the formulation was vortexed extensively, while the temperature was allowed to increase from ~2°C to above the cloud point." (*See* paragraph spanning pages 32-33.) This is contrary to the assertion that the temperature does not need to be raised above the cloud point to obtain particles. (OA at page 3.) Evans is clear that temperature cycling was "repeated several times, until the

particle size of the formulation was in the range of 200-500 nm, as measured by dynamic light scattering." (page 33, lines 5-6.) Thus, Evans not only desired the formation of a particles, but the particle had to achieve a particular size. To do this Evans performed temperature cycling with the mixture of DNA, BAK and CRL1005. In addition, Evans does not teach cold filtering of the mixture to produce a sterile formulation. Therefore, Evans is deficient as a primary reference upon which to base a *prima facie* case of obviousness.

These deficiencies are not cured by the disclosure of Hunter. Hunter does not disclose, suggest or otherwise contemplate a method of producing a sterile cationic surfactant, block copolymer and polynucleotide formulation without the need for thermal cycling. As such, taken together Evans and Hunter do not teach all elements of the claimed invention. The invention is drawn to mixing a polynucleotide, a cationic surfactant and a copolymer at a temperature below the cloud point of the copolymer and filtering the combined mixture. Applicants respectfully assert that this combination of references is insufficient to establish a *prima facie* case of obviousness and respectfully request that the Examiner reconsider and withdraw the rejection.

The art teaches away from filtering a DNA and cationic surfactant mixture

The cited reference teaches away from sterile filtering a combination of cationic surfactant, DNA and copolymer mixture. Evans teaches that the combination of BAK and DNA forms complexes or precipitates that are to large to filter. (See Evans page 2, lines 27-28; citing WO 99/21591) Specifically, the incorporated reference WO 99/21591 (**EXHIBIT A**) teaches complexing BAK and DNA for the purpose of formulating a composition that can be used to introduce DNA into a host or host cell. In order to

prepare a sterile formulation, that may be administered to an animal, the DNA and BAK stock solution are filtered separately before the DNA and BAK are combined. (*See* Exhibit A, Example 4.) WO 99/21591 teaches that once DNA and BAK is combined it will either form a vesicular complex or a precipitate in an aqueous solution. BAK alone does not form a vesicular structure or precipitate in aqueous solution and neither does DNA. Depending on the concentration of BAK in the BAK-DNA mix, either a vesicular complex ranging in size from 50-400 nm will form or the mixture will form a snowy flocculent precipitate. (*See* Exhibit A, Example 2.). Thus, at the time the invention was made the ordinary artisan would have expected that a combination of cationic surfactant and DNA will result in the formation of vesicles and/or precipitates that cannot be filtered when combined together and that the DNA and cationic surfactant would have to be filtered separately.

Obviousness cannot be predicated on what is not known at the time an invention is made, even if the inherency of a certain feature is later established. *In re Rijckaert*, 9 F.2d 1531 (Fed. Cir. 1993). In a post filing date reference by Evans (**EXHIBIT B**) the author acknowledged that "[p]lasmid DNA and BAK surfactants form precipitates that have been reported to enhance DNA delivery. Based on this report, **an unexpected result** from our studies is that DNA-BAK precipitates do not coexist with CRL-1005-BAK-DNA particles (ternary complexes) in these formulations above the cloud point of CRL1005." (*See* Exhibit B, page 1937, column1 , 2nd paragraph) (emphasis added). The post filing date reference establishes that Evans in the International Published Application No. WO 02/00844 did not appreciate that the mixture of DNA-BAK-CRL1005 does not form precipitates.

Even after making the observation that the mixture of DNA, BAK and CRL1005 does not form precipitates above the cloud point, Evans would not make a sterile solution by filtering the combined mixture below the clout point. "Preparation of sterile vaccine formulations requires only the addition of sterile BAK to a solution of DNA/CRL1005 that is sterile filtered below the cloud point." (*See* Exhibit B, page 1937, column1 , 3rd paragraph.) Thus, Evan would filter two components the DNA and CRL1005 mixture and the BAK solution separately before mixing them. This is differs from the claimed invention which uses only a single filtration step.

Applicants have discovered that the process of making a sterile polynucleotide solution can be simplified by combining BAK-DNA-copolymer below the cloud point of the copolymer and sterile filtering the mixture, packaging the mixture and storing the mixture without raising the temperature above the cloud point at any point during the process. (*See* paragraph [0067].) Contrary to the Examiner assertion the ordinary artisan would not have been motivated to combine the teachings of Evans and Hunter to arrive at the instantly claimed method of producing a sterile formulation of cationic surfactant, polynucleotide and copolymer. The ordinary artisan at the time the invention was made did not know that a combination of DNA-BAK in the presence of a copolymer does not form the expected BAK-DNA precipitate. As such, Applicants respectfully assert that a *prima facie* case of obviousness has not been established and respectfully request that the Examiner reconsider and withdraw the rejection.

Unexpected results

Applicants have unexpectedly discovered that microparticle formation does not need to occur prior to sterilization and storage at -80°C. Applicants discovered that the

mixture of DNA, BAK and copolymer can be sterile filtered below the cloud point of the solution, and that the mixture can be aliquoted into sterile vials before frozen storage. (*See* paragraph [0067].) Thus, Applicants discovery that the combination of polynucleotide, cationic surfactant and copolymer mixture can be filtered is unexpected because it was not known that a DNA and cationic surfactant mixture does not form a precipitate in this combination. Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

Claim 9

The Examiner has also rejected claim 9 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Evans and Hunter as applied to claims 1-8 and 11-22 above, and further in view of U.S. Patent 6,933,286 ("Emanuele"). Evans teaches formulating DNA vaccines by mixing cationic surfactant BAK, a POP-POE copolymer (such as CRL 1005) and a polynucleotide at a temperature below the cloud point of the copolymer (2-7°C). (OA at page 2) The Examiner asserts "[t]hus Evans does not require "temperature cycling" through the cloud point" (OA at page 3.) "Evans performed temperature cycling in order to adjust the size of the particles formed by mixing the cationic surfactant." (OA at page 3.) "However, it is clear from the disclosure of Evans that the formation of the particle is what is desired, and that temperature cycling is not necessary to obtain it." (OA at page 3.) Evans did not teach a cold filtration step, this step is taught by Hunter and the Examiner asserts that this step would be obvious since the composition is intended to be used as a vaccine. Emanuele taught formulations

comprising POP-POE-POP copolymers and nucleic acid delivery vehicles. Applicants respectfully traverse this rejection.

As stated *supra*, Evans and Hunter do not teach all elements of the currently pending claims, the references teach away from the method of producing a sterile polynucleotide solution as claimed, and it is unexpected that the combination of cationic surfactant, polynucleotide and copolymer form a solution that does not form a precipitate, and that the composition can be sterile filtered in this combination. Emanuele does not cure the deficiencies of Evans and Hunter because the combined references still do not teach or suggest the production of cationic surfactant, block copolymers and polynucleotide compositions without the need for thermal cycling. Indeed, Emanuele teaches POP-POE-POP block copolymers and polynucleotide formulations, however, there is no suggestion to add a cationic surfactant. As such, Applicants respectfully assert that a *prima facie* case of obviousness has not been established and respectfully request that the Examiner reconsider and withdraw the rejection.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Exhibit A

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(54) Title: COMPOSITIONS AND METHODS FOR DELIVERY OF GENETIC MATERIAL

(57) Abstract

A soluble ionic complex is formed by an aqueous mixture of a benzylammonium group–containing surfactant and a polynucleic acid sequence. When the mixture forms a vesicular complex, the sequence is packaged therein. This composition is useful in pharmaceutical compositions and in methods of delivering the polynucleic acid sequence (which preferably encodes a protein or peptide) to a cell for expression. Such methods are useful in therapy, as vaccines and as gene therapy agents.

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COMPOSITIONS AND METHODS FOR DELIVERY OF GENETIC MATERIAL

Field of the Invention

The present invention relates to compositions and methods for introducing genetic material into cells. More particularly, the present invention relates to compositions and methods for in vitro and *in vivo* gene transfer, which can be used to deliver protective and/or therapeutic agents including genetic material that encodes protein targets for immunization and therapeutic proteins.

Background of the Invention

The direct introduction of a normal, functional gene into a living animal has been studied as a means for replacing defective genetic information. DNA can be introduced directly into cells of a living animal using viral vectors, liposomes, lipid complexes, ligand/DNA conjugates, and microprojectile bombardment, among other methods. Various methods and compositions for mediating transfer of DNA to cells *in vivo* and/or *in vitro* are referred to in U. S. Patent No. 5,593,972, issued January 14, 1997; U. S. Patent No. 5,580,859, issued December 3, 1996; U. S. Patent No. 5,589,466 issued December 31, 1996; U. S. Patent No. 5,676,954, issued November 19, 1996; International Patent Publications Nos. WO90/11092, published March 21, 1990; WO93/17706, published March 10, 1993; WO93/23552, published May 21, 1993; and WO94/16737, published January 26, 1994, and the priority applications cited therein.

Despite the knowledge extant in the art, there remains a need for improved methods of DNA transfer, as well as for improved methods and compositions for *in vivo* and *in vitro* nucleic acid transfer. There remains a need for improved methods of drug delivery.

Summary of the Invention

In one aspect, the invention provides soluble, ionic complex comprising an aqueous mixture of a benzylammonium group-containing surfactant and a polynucleic acid sequence. In one embodiment, the complex is a vesicular-like or liposomal-like complex comprising an aqueous mixture of a benzylammonium group-containing surfactant of the formula described herein and a polynucleic acid sequence, with the sequence substantially packaged in the vesicular complex.

In another aspect, the invention provides a mixture of multiple ionic and/or vesicular complexes of uniform size, as above described. In one embodiment, the composition is formed by mixing an aqueous solution of a benzylammonium-containing surfactant, preferably benzalkonium chloride, with a polynucleic acid sequence.

In still another aspect, the invention provides a pharmaceutical composition comprising at least one, and preferably multiple ionic complexes or vesicular complexes described above and a suitable pharmaceutical carrier.

In yet another aspect, the invention provides a method of introducing a polynucleic acid sequence into a cell comprising the step of contacting said cell with the above described complexes or compositions containing them.

In another aspect, the invention provides a method of facilitating the uptake of a polynucleic acid sequence into a cell comprising contacting the cell with a soluble ionic complex described above, or with a polynucleic acid substantially packaged in a vesicular complex formed by an aqueous mixture of a benzylammonium-containing surfactant with the polynucleic acid sequence.

In a further aspect, the invention provides methods of inducing an immune response in a mammalian or vertebrate subject to a pathogenic antigen or disease, which methods include the step of administering to cells of said subject, an effective amount of a complex as described herein, wherein the polynucleic acid sequence encodes at least one epitope that is identical or substantially similar to an epitope of a antigen of said pathogen, or a sequence encoding a target protein, said protein comprising an epitope identical or substantially similar to an epitope of a protein associated with cells that characterize said disease. The epitope or protein-encoding

sequence is under the control of regulatory sequences that direct expression of said protein in the cells of said subject.

In still another aspect, the invention provides a method of treating a mammalian or vertebrate subject for a disease comprising the step of administering to cells of said subject, an effective amount of a composition comprising a complex of this invention formed by an aqueous mixture of a benzylammonium-containing surfactant and a polynucleic acid sequence, wherein said polynucleic acid sequence comprises a sequence which encodes a protein that produces a therapeutic effect on the subject or a protein that compensates for a missing, non-functional or partially functioning native mammalian protein, the protein-encoding sequence under the control of regulatory sequences that direct expression of said protein in the cells of said subject.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 is a bar graph showing the humoral (antibody) response measured in optical density (OD) at 450 nm in serum of individual Balb/C mice to an aqueous composition of the invention containing the indicated concentrations of benzalkonium chloride and indicated amounts of a DNA plasmid encoding the gD_2 protein of Herpes Simplex Virus as measured by standard ELISA. The positive and negative controls are the same plasmid with no transfection facilitating agent (DNA only) and the plasmid with no gD_2 encoding sequence with no transfection facilitating agent (023ctrl). Each bar represents a single animal. See Example 4 below.

Fig. 2 is a bar graph showing group average humoral (antibody) responses in the animals of Fig. 1. The responses are measured according to Antibody Response Calculations in ng/ml, as defined in Example 4 below.

Fig. 3 is a scatter plot graph showing the individual (animals represented by ◇, □, △, X, and ∗) and group average (represented by •) cellular responses of the animals of Fig. 1. Systemic cellular response (SI) was measured using a splenic cell proliferation assay.

Detailed Description of the Invention

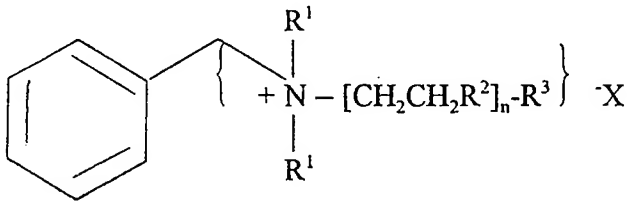
The present invention provides a novel soluble ionic complex comprising an aqueous mixture of a benzylammonium group-containing surfactant; and a polynucleic acid sequence. These soluble complexes may be in the form of vesicular complexes containing polynucleic acid sequences substantially packaged therein. Compositions containing the complexes are useful as pharmaceutical (therapeutic and vaccinal) agents and as gene therapy reagents in methods for introducing the polynucleic acid sequences into a cell for a variety of therapeutic and prophylactic purposes, as well as for research purposes. The compositions and methods of the present invention provide for a high level of uptake and function of the polynucleic acid sequences and molecules.

I. The Soluble Ionic Complexes

A soluble ionic complex of this invention is formed by an aqueous mixture of a benzylammonium group-containing surfactant and a polynucleic acid sequence. The combination of the surfactant and the polynucleic acid sequence forms a vesicular-like or liposomal-like structure, in which substantially all of the polynucleic acid sequence becomes packaged. Minor amounts of the polynucleic acid sequence are associated with the exteriors of the vesicular complex. Without wishing to be bound by theory, these complexes appear to provide *in vivo* stability to the polynucleic acid sequences associated therewith, and thus facilitate transfection of such sequences into host cells.

A. The Surfactant

The benzylammonium group-containing surfactant is preferably a surfactant of the formula:



wherein X is an anion;

each R^1 is independently a hydrogen or a lower alkyl group comprising from 1 to 6 carbon atoms;

R^2 is CH_2 or -O-;

R^3 is H, CH_3, C_2H_5, phenyl, mono-substituted phenyl, or di-substituted phenyl, wherein said substitutions are independently selected from among C_1-C_{10} branched or straight chain alkyls groups; and

n is an integer of 2 through 7, provided that when n is 1, R^3 is methyl, ethyl, phenyl or substituted phenyl; when n is 4 through 6, R^3 is H, methyl, ethyl or phenyl; when n is 6, R^3 is H, methyl or ethyl; and when n is 7, R^3 is H or methyl.

Examples of preferred benzylammonium-group containing surfactants include those which comprise a dimethyl benzyl ammonium group linked to an alkyl group or an alkyl group linked to an aromatic group. The anion is selected from among anions that results in soluble complexes with the polynucleic acid sequence in water. In some embodiments, the anion is a halide, a sulfate, or a carbonate. In one preferred embodiment, the anion in the surfactant is a halide, such as chloride. One of skill in the art may select from among a number of suitable anions for the preparation of a surfactant suitable for the present invention.

One presently preferred example of a benzylammonium group-containing surfactant is a benzalkonium halide, such as benzalkonium chloride. Benzalkonium chloride is a cationic surfactant known to condense DNA [V. Jelen et al, Journal of Electroanalytical Chemistry, 377:197-203 (1994)]. It has also been used as an antimicrobial agent for parenteral preparations [*Remington's Pharmaceutical Sciences*, A. Osol, a standard reference text in this field; and "Pharmaceutical Dosage Forms: Parenteral Medication", Vol.I, K. Avis et al (eds), Marcel Dekker, Inc., New York (1992)]. Benzalkonium chloride refers to commercially available surfactants which are a mixture of alkyldimethylbenzylammonium chlorides of the formula above where $[CH_2CH_2R^2]_n$-R^3 is a mixture of alkyls C_8H_{17} to $C_{18}H_{37}$, i.e., n is 2-6, each R^2 is CH_2 and R^3 is hydrogen, methyl or ethyl. Benzalkonium chloride which may be used as the surfactant in the complexes, compositions, kits and methods of the invention

may be homogenous or may contain a mixture of compounds characterized by having two or more different R groups according to the formula above. Benzalkonium chloride, U.S.P. grade can be purchased from Spectrum Chemical Mfg. Corp., Gardena, CA.

Another exemplary surfactant useful in this invention is a benzethonium halide, such as benzethonium chloride. Benzethonium chloride refers to a commercially available surfactant N,N-dimethyl-N-[2- [2-[4 (1,1,3,3-tetramethylbutylphenoxy)ethoxy]ethyl]ammonium chloride, and is described in U. S. Patent Nos. 2,115,250, 2,170,111 and 2,229,024. It has also been used as an antimicrobial agent for parenteral preparations [Pharmaceutical Dosage Forms, cited above]. Benzethonium chloride has the formula set out above where n is 2 and R^3 is 4-[1,1,3,3-tetra methylbutyl]phenyl. Benzethonium chloride, U.S.P. grade can be purchased from Spectrum Chemical Mfg. Corp., Gardena, CA.

The complexes of the present invention comprise a final concentration of benzylammonium-group containing surfactant between about 0.001 to about 2.4% by volume (w/v). Desirably, the complexes have a final concentration of the surfactant of between about 0.001-0.10% w/v. More preferably, the concentration of surfactant in the complexes is between about 0.005-0.06% w/v. A particularly desirable vesicular complex of this invention contains a benzylammonium-group containing surfactant in a concentration of between about 0.005 -0.03%w/v. Manipulation of the other components of the complex, e.g., the polynucleic acid, and the buffering agents and optional isotonicity and pH adjusting agents, can reduce the toxicity of the surfactant, and permit a soluble complex to be formed at a variety of concentrations of surfactant without encountering precipitation. One of skill in the art given the instructions provided herein is expected to be able to readily manipulate the components of this invention to provide such soluble complexes at a variety of surfactant concentrations.

B. The Polynucleic Acid Sequence

Another component of the complexes of the present invention is a polynucleic acid sequence , which when admixed with the above-described aqueous surfactant, forms a soluble ionic complex therewith. In preferred embodiments, the

polynucleic acid sequence becomes substantially packaged in the vesicular-like or liposomal-like complex, and only a minor amount of such polynucleic acid sequence is associated with the exterior of the complex.

The polynucleic acid sequences which form part of the complexes of this invention are preferably "dissociated from an infectious agent", i.e., are not part of a viral, bacterial or eukaryotic vector, either active, inactivated, living or dead, that is capable of infecting a cell. In some embodiments, the polynucleic acid sequence present in compositions of the present invention are preferably free from infectious agents such as viral particles, particularly retroviral particles, and are preferably non-infectious plasmid DNA molecules. In some preferred embodiments, the compositions are free of lipids, such as cationic lipids, and/or other surfactants, and/or local anaesthetics. In some embodiments, the polynucleic acid sequences are free from the precipitating agent $CaPO_4$.

The complexes and compositions of the present invention preferably comprise between about 10 μg/ml to about 20 mg/ml of polynucleic acid sequences or molecules. Preferably, the aqueous compositions of surfactant and polynucleic acid sequences which form the complexes of the invention comprises a concentration of polynucleic acid sequences of between about 50 μg/ml to about 10 mg/ml of polynucleic acid sequences or molecules. In other preferred embodiments, the aqueous compositions of surfactant and polynucleic acid sequences which form the complexes of the invention comprises a concentration of polynucleic acid sequences of between about 100 μg/ml to about 1 mg/ml of polynucleic acid sequences or molecules.

For example, one embodiment of the complexes of the inventions contains about 0.1-5.0 mg/ml polynucleic acid in a final concentration of 0.010-0.030% w/v benzylammonium-group containing surfactant. Some preferred embodiments comprise 0.010% w/v benzylammonium-containing surfactant and 0.1 mg/ml polynucleic acid molecules. Other preferred embodiments comprise 0.010% w/v benzylammonium-group containing surfactant with about 0.5 mg/ml nucleic acid

molecules. Still other preferred embodiments comprise 0.020% w/v benzylammonium-group containing surfactant with about 0.5 mg/ml nucleic acid molecules.

A particularly desirable embodiment of the complexes of the present invention is formed by between about 100-500 µg DNA molecules at a concentration of 0.1-0.5 mg/ml in a final concentration of 0.010- 0.030% w/v benzalkonium chloride or benzethonium chloride. Another preferred embodiment comprises 0.010% w/v benzalkonium chloride and 0.1 mg/ml nucleic acid molecules. Still other preferred embodiments comprise 0.010% w/v benzalkonium chloride and about 0.5 mg/ml nucleic acid molecules. Some preferred embodiments comprise 0.020% w/v benzalkonium chloride and 0.5 mg/ml nucleic acid molecules. Some preferred embodiments comprise 0.010% w/v benzethonium chloride and 0.1 mg/ml nucleic acid molecules. Some preferred embodiments comprise 0.010% w/v benzethonium chloride and 0.5 mg/ml nucleic acid molecules. Some preferred embodiments comprise 0.020% w/v benzethonium chloride and 0.5 mg/ml nucleic acid molecules.

The polynucleic acid sequence of this invention may be any nucleic acid sequence and may take a variety of known forms, as taught elsewhere in the art. Thus, as used herein, the terms "polynucleic acid sequence", "nucleic acid molecule", "polynucleotide", "DNA construct", "genetic construct" and "nucleotide sequence" are interchangeable. Polynucleic acid sequences of this invention can be deoxyribonucleic acid sequences (DNA) and/or ribonucleic acid sequences (RNA). These nucleic acid sequences or molecules may be cDNA, genomic DNA, synthesized DNA, DNA molecules or plasmids or a hybrid thereof, or an RNA molecule such as mRNA. The polynucleic acid sequence may also encode antisense sequences which inhibit gene expression of genes whose expression is undesirable. A polynucleic acid molecule may serve as a template for antisense molecules and ribozymes and such sequences may be preferably linked to regulatory elements necessary for production of sufficient copies of the antisense and ribozyme molecules encoded thereby respectively or a ribozyme.

Polynucleic acid sequences or molecules useful in the present invention may serve a variety of functions, but are essentially provided to a selected host cell for a multitude of known therapeutic, prophylactic, and research uses. For example, the

sequences are useful in the complexes of the invention as: 1) sequences encoding for proteins that function as prophylactic and/or therapeutic immunizing agents; 2) replacement copies of defective, missing or non-functioning genes; 3) sequences encoding therapeutic proteins; 4) antisense sequences or sequences encoding for antisense molecules; or 5) sequences encoding for, or genetic templates for, ribozymes.

Thus, in desired embodiments, the polynucleic acid sequence or molecule may comprise a sequence that encodes a peptides or protein. The sequence may be a plasmid which comprises a nucleotide sequence that encodes a protein or peptide, the encoding sequence operably linked to regulatory sequences directing expression of the protein or peptide in a host cell. Such regulatory sequences direct replication, transcription, translation and/or expression of the encoded protein or peptide in selected host cells, e.g., mammalian or vertebrate cells. As used herein, the term "expressible form" refers to polynucleic acid sequences or gene constructs which contain the necessary regulatory elements operably linked to a coding sequence that encodes a target protein, such that when present in the host cell, the coding sequence will be expressed.

The regulatory elements necessary for expression of a sequence encoding a protein or peptide include a promoter (constitutive or inducible), an initiation signal or codon, a termination signal or stop codon, and a polyadenylation signal. In addition, enhancers are often required, as well as other sequences, e.g., a Kozak region, etc. Such regulatory elements may be selected from among those known to be preferred in a selected host cell, and the polynucleic acid sequence may likewise contain codons which are known to be preferentially expressed in certain host cells. Such regulatory elements are operable in the cell of a mammalian or vertebrate subject or tissue to whom they are administered. Initiation codons and stop codon are generally considered to be part of a nucleotide sequence that encodes the desired protein. However, it is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the host cells. Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine or gene therapy vector, include but are not limited to, promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human actin, human myosin, human hemoglobin, human muscle creatine and human metallothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal which is in pCEP4 plasmid (Invitrogen, San Diego, CA), referred to as the SV40 polyadenylation signal, is used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the polynucleic acid sequence of the complex. Such additional elements include enhancers, such as those selected from the group including but not limited to: human actin, human myosin, human hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV. The polynucleic acid sequences of this invention may also include a mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in a mammalian or vertebrate cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, CA) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

Additionally, polynucleic acid sequence (e.g., DNA) which is useful to promote integration of the polynucleic acid sequence into the chromosome of the cell may also be included in the polynucleic acid sequence or DNA molecule useful in this invention. One embodiment of a polynucleic acid sequence is as a linear minichromosome including a centromere, telomeres and an origin of replication.

The polynucleic acid sequence may also contain an additional element which serves as a target for cell destruction if it is desirable to eliminate cells receiving the sequence for any reason. A herpes thymidine kinase (tk) gene in an expressible form can be included in the polynucleic acid molecule of the complex. Upon administration of the drug gangcyclovir, any cell transfected with the complex and thus producing tk, will be selectively killed. Thus, the polynucleic acid sequence can provide the means for the selective destruction of cells transfected therewith.

Depending on the use to which the complex is applied, the polynucleic acid sequence may encode a wide variety of peptides or proteins useful in pharmaceutical reagents and in research. As one example, the proteins and/or peptides encoded by the polynucleic acid sequence of the vesicular complex can include a target protein useful to induce or elicit a therapeutic or prophylactic immune response. The target protein is an immunogenic protein which shares at least an epitope with a protein from the pathogen (e.g., a virus, a bacterium, yeast, parasite, etc) or from an undesirable cell-type such as a cancer cell or a cell involved in autoimmune disease against which immunization is required. The protein can be an epitope identical or substantially similar to an epitope of a antigen of the pathogenic microorganism or undesirable cell type. As used herein, the term "substantially similar epitope" is meant to refer to an epitope that has a structure which is not identical to an epitope of a protein but nonetheless invokes a cellular or humoral immune response which cross reacts to that protein. The protein can be an epitope identical or substantially similar to an epitope of a protein associated with, e.g., hyperproliferating cells; or an epitope identical or substantially similar to an epitope of a protein associated with, e.g, cells that characterize an autoimmune disease.

The polynucleic acid sequence may also encode a therapeutic or compensating protein, i.e., it can encode a protein or peptide which can compensate for a protein product that is deficient, missing, nonfunctional or partially functioning, endogenously produced, in a cell or mammalian or vertebrate subject due to an absent, defective, non-functioning or partially functioning endogenous gene. The polynucleic

acid sequence can also encode a protein or peptide that produces a therapeutic effect in a mammalian or vertebrate subject.

Exemplary protein products can readily be selected by one of skill in the art for insertion into a host cell. Among the non-exclusive lists of protein-encoding polynucleic acid sequences are sequences from an oncogene selected from the group consisting of *myb, myc, fyn, ras, sarc,* neu and trk. The sequences can also encode a protein product of the translocation gene *bcl/abl*; a protein product of P53; or for example, the protein EGRF. Still other exemplary polynucleic acid sequences useful in various aspects of this invention can encode a variable region of an antibody made by a B cell lymphoma; a variable region of a T cell surface receptor of a T cell lymphoma; a variable region of an antibody involved in B cell mediated autoimmune disease; and a variable region of a T cell surface receptor involved in T cell mediated autoimmune disease.

Thus, any polynucleic acid sequence which is desired to be inserted in a selected host cell can form part of the vesicular-like or liposomal-like complex of the present invention. One of skill in the art of therapeutics, vaccines and gene therapy may readily select and incorporate a desired polynucleic acid sequence using the teachings of the present invention.

C. The Buffer and Other Reagents in the Complex

The aqueous mixture of the benzylammonium-containing surfactant and the polynucleic acid sequence which form the soluble ionic complexes or vesicular complexes of the invention may also contain other optional agents, such as aqueous buffering agents, isotonicity adjusting agents, and pH adjusting agents. Suitable buffers for use in forming the complexes may be conventionally selected from among many known buffers used in the formation of pharmaceutical products. Among a non-exclusive list of buffers are phosphate buffers, such as phosphate buffered saline and citrate buffers. Selection of such buffers is clearly within the skill of the art. Preferably, the aqueous mixture which forms the complexes contains a buffer in a concentration of about 2 to about 50 mM, preferably about 5 to 30 mM. In one embodiment, the composition contains about 5mM phosphate buffered saline.

The compositions of the present invention may also preferably contain isotonicity adjusting agents. For example, for pharmaceutical compositions for parenteral administration, especially intramuscularly, subcutaneously and intradermally, the aqueous mixture forming the complexes is desirably isotonic. However, as desired, one of skill in the art may readily make the compositions hypotonic or hypertonic. Some examples of typical tonicity adjusting agents include, without limitation, sodium chloride, sucrose, mannitol, sorbitol, and trehalose. For example, where the complex is desirably hypotonic to isotonic, an tonicity adjusting agent, e.g., sucrose, is present in the aqueous admixture forming the complex in a concentration of 0 to about 9.25% w/v. For example, where the complex is desirably hypertonic, an tonicity adjusting agent, e.g., sucrose, is present in the aqueous admixture forming the complex in a concentration of greater than 9% w/v. One of skill in the art of pharmaceutical preparation can readily adjust this characteristic of the complex.

Similarly, ionic strength of the complex may be adjusted by one of skill in the art by the addition of charged molecules, such as sodium chloride. In the examples below, sodium chloride is present at a concentration of between about 0 - 0.9% w/v.

The aqueous mixture forming the complexes of this invention are preferably characterized by a pH between about 6.0 to about 8.0. More preferably, a desirably pH is about 6.7 ± 0.5. Suitably pH adjustments may be readily made by a selection of agents and is well within the knowledge of one skilled in the pharmaceutical arts.

D. Examples of Complexes of the Invention

According to one embodiment of the invention, a soluble ionic complex is formed by an isotonic, aqueous admixture benzalkonium chloride and plasmid DNA, such as illustrated in Examples 1 and 4 below. In another example of the invention illustrated below, the complex of this invention is formed from an aqueous, isotonic mixture of benzethonium chloride and plasmid DNA, as illustrated in Example 5. Such compositions comprise the polynucleic acid sequence substantially packaged in the vesicular complex formed by an aqueous mixture of a benzylammonium-containing

surfactant and the sequence, as demonstrated by Examples 2 and 3. Similarly, other compositions of this invention contain multiple vesicular complexes of uniform size, each vesicular complex containing polynucleic acid sequence substantially packaged in the complexes formed by admixing an aqueous mixture of the benzylammonium-containing surfactant and a solution containing the polynucleic acid sequence. The method of preparing such complexes of the invention is described in detail in Example 1 below.

The compositions of this invention, i.e., the complexes formed by the benzylammonium-group containing surfactants and polynucleic acid sequences, increase and/or facilitate uptake and/or expression of the polynucleic acid sequences by host cells, compared to the uptake or expression which occurs when the identical polynucleic acid sequence or molecule is administered to a host cell in the absence of the benzylammonium-group containing surfactants. See the results of Example 3 below.

II. Pharmaceutical Compositions of this Invention

The complexes and compositions of this invention may be employed in pharmaceutical compositions and in methods to introduce polynucleic acid sequences, e.g., genetic material, into cells *in vitro* or *in vivo*. A pharmaceutical composition of this invention comprises the soluble ionic complexes as described above in a suitable pharmaceutical carrier. In some instances, the aqueous buffer may itself be suitable. The vaccines and therapeutics according to the present invention are formulated according to the mode of administration to be used. One having ordinary skill in the art can readily formulate a pharmaceutical composition that comprises a complex as described above. In cases where intramuscular injection is the chosen mode of administration, an isotonic formulation is preferably used. Generally, additives for tonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vasoconstriction agent is added to the formulation. The pharmaceutical preparations according to the present

invention are provided sterile and pyrogen free. Although for pharmaceutical use, any route of administration may be employed, it is preferred that the composition of the invention be an injectable formulation. In one embodiment of the invention a pharmaceutical composition comprises a vesicular complex which contains the polynucleic acid molecule substantially packaged in the vesicle, with some minor amount of the sequence associated with the exterior of the vesicular complex, with the complex being in a suitable pharmaceutical carrier.

The compositions of the present invention, when used as pharmaceutical compositions, can comprise about 1 ng to about 1000 μg of DNA. In some preferred embodiments, the vaccines and therapeutics contain about 10 ng to about 800 μg DNA. In some preferred embodiments, the vaccines and therapeutics contain about 0.1 to about 500 μg DNA. In some preferred embodiments, the vaccines and therapeutics contain about 1 to about 350 μg DNA. In some preferred embodiments, the vaccines and therapeutics contain about 25 to about 250 μg DNA. In some preferred embodiments, the vaccines and therapeutics contain about 100 μg DNA.

In addition, other agents which may function as transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with the benzylammonium-group containing surfactants-nucleic acid molecule complexes include growth factors, cytokines and lymphokines such as alpha-interferon, gamma-interferon, platelet derived growth factor (PDGF), colony stimulating factors, such as G-CSF, GM-CSF, tumor necrosis factor (TNF), epidermal growth factor (EGF), and the interleukins, such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12. Further, fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicular complexes such as squalene and squalene, and hyaluronic acid may also be used administered in conjunction with the compositions of the invention.

The compositions of the present invention may be combined with collagen as an emulsion and delivered parenterally. The collagen emulsion provides a means for sustained release of DNA. Preferably 50 μl to 2 ml of collagen are used. About 100

μg DNA are combined with 1 ml of collagen in a preferred embodiment using this formulation. Other sustained release formulations such as those described in *Remington's Pharmaceutical Sciences,* A. Osol, a standard reference text in this field. Such formulations include aqueous suspensions, oil solutions and suspensions, emulsions and implants as well as reservoirs and transdermal devices. In some embodiments, time release formulations for compositions of the present invention are provided. In some embodiments, it is preferred that the compositions of the present invention are time released between 6-144 hours, preferably 12-96 hours, more preferably 18-72 hours.

III. Methods of Use of the Complexes

A. In Vitro Transfection Methods

The invention provides *in vitro* transfection methods using the complexes and compositions comprising the aqueous mixtures of polynucleic acid sequences and benzylammonium-group containing surfactants as above described. A method of the invention facilitates the uptake of a polynucleic acid sequence into a cell comprising the step of contacting the cell with a soluble ionic complex comprising an aqueous mixture of a benzylammonium group-containing surfactant and a polynucleic acid sequence, as described above. According to this *in vitro* method, the complex may be introduced into tissue cultures or cells in solution in the form of a vesicular complex in which the polynucleic acid sequence is substantially packaged in the complex, with a minor amount of sequence associated with the exterior of the complex.

B. Methods of In Vivo Administration

The invention also provides *in vivo* therapeutic, prophylactic and gene therapy methods of transferring polynucleic acid sequences (e.g., genetic material) into cells of a mammal or a vertebrate using the pharmaceutical compositions and complexes of this invention. The benzylammonium-group containing surfactants are administered as a mixture with the nucleic acid molecule. In preferred embodiments, the benzylammonium-group containing surfactants are mixed with nucleic acid

molecules to form vesicular complexes. The methods of this invention involve the step of administering to a cell or tissue of the mammal or vertebrate, a composition comprising a benzylammonium-group containing surfactant and a polynucleic acid sequence. Transfection of the polynucleic acid sequence, e.g., the DNA or RNA molecule in the surfactant:polynucleic acid sequence complex, into a living cell results in the expression of the DNA or RNA. Where the DNA or RNA encode a desired protein, the desired protein is produced. When taken up by a cell, the nucleotide sequence encoding the desired protein operably linked to the regulatory elements may remain present in the cell as a functioning extrachromosomal molecule or it may integrate into the cell's chromosomal DNA. The complex may be introduced into cells and the polynucleotide may remains as separate genetic material in the form of a plasmid. Alternatively, the complex can be employed to introduce a linear polynucleic acid sequence (DNA) into the cell, which DNA can integrate into the chromosome. When introducing the complex into the cell, reagents which promote DNA integration into chromosomes may be added.

i. Inducing Immune Responses and Therapeutic Treatment

According to some aspects of the present invention, compositions and methods are provided which prophylactically and/or therapeutically immunize an individual against a pathogen, allergen or abnormal, disease-related cell. In one embodiment, the invention provides a method of inducing an immune response in a mammalian or vertebrate subject, preferably a human, to a pathogenic antigen comprising the step of administering to cells of said subject, an effective amount of a composition or soluble complex as described above. In this complex, the polynucleic acid sequence comprises a sequence which encodes at least one epitope that is identical or substantially similar to an epitope of an antigen of the pathogen against which an immune response can be generated which will be directed against the target pathogen antigen, allergen or antigen of an abnormal and/or disease-related cell. The polynucleic acid sequence can also encode a peptide or protein which is immunologically cross reactive to the target pathogen antigen, allergen or antigen of an abnormal and/or disease-related cell. The epitope-encoding sequence, which is part of the

polynucleotide sequence, is under the control of regulatory sequences that direct expression of said protein in the cells or tissue of the mammalian or vertebrate subject.

In a similar embodiment, the invention provides a method of immunizing a mammalian or vertebrate subject against a disease comprising the step of administering to said subject a composition comprising an effective amount of a composition comprising a complex of this invention, where the polynucleic acid sequence comprises a nucleotide sequence encoding a target protein, operatively linked to regulatory sequences directing the expression of said protein in the cells of said subject. The target protein can be an epitope identical or substantially similar to an epitope of a protein associated with cells that characterize the disease.

In other aspect, the method of the invention permits therapeutic treatment of a mammalian or vertebrate subject for a disease comprising the step of administering to cells of said subject, an effective amount of a composition comprising a complex described above. In the complexes useful in this aspect of the invention, the polynucleic acid sequence comprises a sequence which encodes a protein or peptide that produces a therapeutic effect on the subject, said protein-encoding sequence under the control of regulatory sequences that direct expression of said protein in the cells of said subject.

According to these aspects of the present invention, the DNA or RNA that encodes a desired protein is introduced into the cells of an individual where it is expressed, thus producing the desired protein. In such embodiments, an immune response is generated that is immunologically cross reactive with a pathogen antigen, allergen or antigen of the abnormal and/or disease-related cell. The resulting immune response is broad based: in addition to a humoral immune response, immune responses from both arms of the cellular immune response are elicited. The methods of the present invention are useful for conferring prophylactic and therapeutic immunity. Thus, a method of immunizing includes both methods of protecting an individual from pathogen challenge, or occurrence or proliferation of specific cells as well as methods of treating an individual suffering from pathogen infection, hyperproliferative disease or autoimmune disease with which the target protein is associated.

This aspect of the method of the present invention is useful to immunize individuals against pathogenic agents and organisms such that an immune response against a pathogen protein provides protective immunity against the pathogen. The present invention is useful to combat hyperproliferative diseases and disorders such as cancer by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. The present invention is useful to combat autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition.

In some preferred embodiments related to immunization applications, the genetic construct contains nucleotide sequences that encode a target protein and further include genes for proteins which enhance the immune response against such target proteins. Examples of such genes are those which encode cytokines and lymphokines such as those listed above in Part II. In some embodiments, it is preferred that the gene for B7.2 and/or GM-CSF is included in genetic constructs used in immunizing compositions.

The present invention may be used to immunize an individual against all pathogens such as viruses, prokaryote and pathogenic eukaryotic organisms such as unicellular pathogenic organisms and multicellular parasites. The present invention is particularly useful to immunize an individual against those pathogens which infect cells and which are not encapsulated, such as viruses, and prokaryote such as gonorrhoeae, listeria and shigella. In addition, the present invention is also useful to immunize an individual against protozoan pathogens which include a stage in the life cycle where they are intracellular pathogens. As used herein, the term "intracellular pathogen" is meant to refer to a virus or pathogenic organism that, for at least part of its reproductive or life cycle, exists within a host cell and therein produces or causes to be produced, pathogen proteins. One of skill in the art, given this disclosure can readily select viral families and genera, or pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites, for which vaccines according to the present invention can be made. See, e.g., the tables of such pathogens

in general immunology texts and in U. S. Patent No. 5,593,972. In some preferred embodiments, the methods of immunizing an individual against a pathogen are directed against HIV, HTLV or HBV.

Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The polynucleic acid sequence used in a composition such as a genetic vaccine employing a complex of this invention can include genetic material which encodes many pathogen antigens. For example, several viral genes may be included in a single construct thereby providing multiple targets. In addition, multiple inoculants which can be delivered to different cells in an individual can be prepared to collectively include, in some cases, a complete or, more preferably, an incomplete such as a near complete set of genes in the vaccine. For example, a complete set of viral genes may be administered using two constructs which each contain a different half of the genome which are administered at different sites. Thus, an immune response may be invoked against each antigen without the risk of an infectious virus being assembled. This allows for the introduction of more than a single antigen target and can eliminate the requirement that protective antigens be identified.

Another aspect of the present invention provides a method of conferring a broad based protective immune response against hyperproliferating cells that are characteristic in hyperproliferative diseases and to a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of complexes of this invention serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein. As used herein, the term "hyperproliferative diseases" is meant to refer to those diseases and disorders characterized by hyperproliferation of cells. Examples of hyperproliferative diseases include all forms of cancer and psoriasis. It has been discovered that introduction of a genetic construct that includes a nucleotide sequence which encodes an immunogenic "hyperproliferating cell-associated protein" into the cells of an individual results in the production of those proteins in the

vaccinated cells of an individual. As used herein, the term "hyperproliferative associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease. To immunize against hyperproliferative diseases, a complex of the invention that includes a polynucleic acid sequence which encodes a protein that is associated with a hyperproliferative disease is administered to an individual.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides which comprise at least an epitope found on such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein which is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein. Such target proteins include those which are proteins encoded by oncogenes such as *myb, myc, fyn,* and the translocation gene *bcr/abl, ras, src,* P53, *neu, trk* and EGRF.

In addition to oncogene products as target antigens, target proteins for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are also used target antigens for autoimmune disease. Other tumor-associated proteins can be used as target proteins such as proteins which are found at higher levels in tumor cells including the protein recognized by monoclonal antibody 17-1A and folate binding proteins.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to prophylactically immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse. Developments in genetics and technology as well as epidemiology allow for the determination of probability and risk assessment for the development of cancer in

individual. Using genetic screening and/or family health histories, it is possible to predict the probability a particular individual has for developing any one of several types of cancer.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer or are otherwise in remission are particularly susceptible to relapse and reoccurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat a recurrence. Thus, once it is known that an individual has had a type of cancer and is at risk of a relapse, they can be immunized in order to prepare their immune system to combat any future appearance of the cancer.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies. T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include Vβ-3, Vβ-14, Vβ-17 and Vα-17. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA [Howell, M.D *et al*, Proc. Natl. Acad. Sci. USA, 88:10921-10925 (1991); Paliard, X. *et al.*, Science, 253:325-329 (1991); Williams, W.V.*et al.*, J. Clin. Invest.,90:326-333 (1992)]. In MS, several specific variable regions of TCRs which are involved in the disease have been characterized. These

TCRs include Vβ-7 and Vα-10. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS [Wucherpfennicf, K.W., *et al.*, Science, 248:1016-1019 (1990); Oksenberg, J.R., *et al.*, Nature, 345:344-346 (1990)]. In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include Vβ-6, Vβ-8, Vβ-14 and Vα-16, Vα-3C, Vα-7, Vα-14, Vα-15, Vα-16, Vα-28 and Vα-12. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be taken and the variable region of those TCRs identified using standard techniques. Genetic vaccines using the complexes of this invention can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Graves disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies which bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody. In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Genetic vaccines can be prepared using this information. For example, in the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes DNA constructs that encode the variable

region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, et al. (1987) *Sequence of Proteins of immunological Interest* U.S. Department of Health and Human Services, Bethesda MD. In addition, a general method for cloning functional variable regions from antibodies can be found in Chaudhary, V.K., *et al.*, Proc. Natl. Acad. Sci.USA, 87:1066 (1990).

In some embodiments of the invention, the individual is subject to a single vaccination to produce a full, broad immune response. In some embodiments of the invention, the individual is subject to a series of vaccinations to produce a full, broad immune response. According to some embodiments of the invention, at least two and preferably four to five injections are given over a period of time. The period of time between injections may include from 24 hours apart to two weeks or longer between injections, preferably one week apart. Alternatively, at least two and up to four separate injections are given simultaneously at different sites.

In some embodiments of the invention, a complete vaccination includes injection of a single inoculant which contains a compositions of this invention which includes a polynucleic acid sequence including sequences encoding one or more targeted epitopes.

In some embodiments of the invention, a complete vaccination includes injection of two or more different inoculants into different sites. For example, in an HIV vaccine according to the invention, the vaccine comprises two inoculants in which each one comprises compositions of this invention encoding different viral proteins. This method of vaccination allows the introduction of as much as a complete set of viral genes into the individual without the risk of assembling an infectious viral particle. Thus, an immune response against most or all of the virus can be invoked in the vaccinated individual. Injection of each inoculant is performed at different sites, preferably at a distance to ensure no cells receive both genetic constructs. As a further safety precaution, some genes may be deleted or altered to further prevent the

capability of infectious viral assembly.

ii. Gene Therapy

Other aspects of the present invention relate to gene therapy; that is, to compositions for and methods of introducing nucleic acid molecules into the cells of an individual exogenous copies of genes which either correspond to defective, missing, non-functioning or partially functioning genes in the individual or which encode therapeutic proteins, i.e., proteins whose presence in the individual will eliminate a deficiency in the individual and/or whose presence will provide a therapeutic effect on the individual thereby providing a means of delivering the protein by an alternative means from protein administration. In aspects of the invention relating to gene therapy, constructs with origins of replication including the necessary antigen for activation are preferred. Thus, a method of treating a mammalian or vertebrate subject for a disease comprises administering to cells of said subject, an effective amount of a composition comprising a complex as above-described. The polynucleic acid sequence of the complex which is useful in this method comprises a sequence which encodes a protein that compensates for a missing, non-functional or partially functioning native mammalian protein, said protein-encoding sequence under the control of regulatory sequences that direct expression of said protein in the cells of said subject.

In some of the embodiments of the invention that relate to gene therapy, the gene constructs contain either compensating genes or genes that encode therapeutic proteins. Examples of compensating genes include a gene which encodes dystrophin or a functional fragment, a gene to compensate for the defective gene in patients suffering from cystic fibrosis, an insulin, a gene to compensate for the defective gene in patients suffering from ADA, and a gene encoding Factor VIII. Examples of genes encoding therapeutic proteins include genes which encodes erythropoietin, interferon, LDL receptor, GMCSF, IL-2, IL-4 and TNF. Additionally, genetic constructs which encode single chain antibody components which specifically bind to toxic substances can be administered.

In some preferred embodiments, the dystrophin gene is provided as part of a mini-gene and used to treat individuals suffering from muscular dystrophy. In some preferred embodiments, a mini-gene which contains coding sequence for a partial dystrophin protein is provided. Dystrophin abnormalities are responsible for both the milder Becker's Muscular Dystrophy (BMD) and the severe Duchenne's Muscular Dystrophy (DMD). In BMD dystrophin is made, but it is abnormal in either size and/or amount. The patient is mild to moderately weak. In DMD no protein is made and the patient is wheelchair-bound by age 13 and usually dies by age 20. In some patients, particularly those suffering from BMD, partial dystrophin protein produced by expression of a mini-gene delivered according to the present invention can provide improved muscle function.

In some preferred embodiments, genes encoding IL-2, IL-4, interferon or TNF are delivered to tumor cells which are either present or removed and then reintroduced into an individual. In some embodiments, a gene encoding gamma interferon is administered to an individual suffering from multiple sclerosis.

Antisense molecules and ribozymes may also be delivered to the cells of an individual by introducing genetic material which acts as a template for copies of such active agents. These agents inactivate or otherwise interfere with the expression of genes that encode proteins whose presence is undesirable. Constructs which contain sequences that encode antisense molecules can be used to inhibit or prevent production of proteins within cells. Thus, production proteins such as oncogene products can be eliminated or reduced. Similarly, ribozymes can disrupt gene expression by selectively destroying messenger RNA before it is translated into protein. In some embodiments, cells are treated according to the invention using constructs that encode antisense or ribozymes as part of a therapeutic regimen which involves administration of other therapeutics and procedures. Gene constructs encoding antisense molecules and ribozymes use similar vectors as those which are used when protein production is desired except that the coding sequence does not contain a start codon to initiate translation of RNA into protein. In some

embodiments, it is preferred that the vectors contain an origin of replication and an expressible form of the appropriate nuclear antigen.

Ribozymes are catalytic RNAs which are capable of self-cleavage or cleavage of another RNA molecule. Several different types of ribozymes, such as hammerhead, hairpin, Tetrahymena group I intron, ahead, and RNase P are known in the art [S. Edgington, Biotechnology, 10:256-262 (1992)]. Hammerhead ribozymes have a catalytic site which has been mapped to a core of less than 40 nucleotides. Several ribozymes in plant viroids and satellite RNAs share a common secondary structure and certain conserved nucleotides. Although these ribozymes naturally serve as their own substrate, the enzyme domain can be targeted to another RNA substrate through base-pairing with sequences flanking the conserved cleavage site. This ability to custom design ribozymes has allowed them to be used for sequence-specific RNA cleavage [G. Paolella *et al.*, EMBO J.,1913-1919 (1992)]. It will therefore be within the scope of one skilled in the art to use different catalytic sequences from various types of ribozymes, such as the hammerhead catalytic sequence and design them in the manner disclosed herein. Ribozymes can be designed against a variety of targets including pathogen nucleotide sequences and oncogenic sequences. Certain preferred embodiments of the invention include sufficient complementarity to specifically target the abl-bcr fusion transcript while maintaining efficiency of the cleavage reaction.

iii. Routes of Administration

In any of the above described pharmaceutical methods, the complex may be administered by any suitable route for such therapy. Among such routes are included parenteral routes, such as intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterial, intraoccular, and intrathecal routes of administration. Mucosal routes of administration are also useful, including rectal, vaginal, urethral and intranasal routes. Topical and transdermal administration is also useful for compositions and methods of this invention. Administration by inhalation is also useful. Suppository preparations or other appropriate dosage forms are also useful. Oral administration may also be employed in the methods of this

invention. Preferred routes of administration include intramuscular, intraperitoneal, intradermal and subcutaneous injection.

Compositions of this invention may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". According to some embodiments of the present invention, the complex of this invention is simultaneously administered to an individual intradermally, subcutaneously and intramuscularly using a needleless injection device. Needleless injection devices are well known and widely available. One having ordinary skill in the art can, following the teachings herein, use needleless injection devices to deliver compositions of this invention to cells of an individual. Needleless injection devices are well suited to deliver compositions of this invention to all tissue. They are particularly useful to deliver compositions of this invention to skin and muscle cells, intradermally, subcutaneously and intramuscularly. In some embodiments, a needleless injection device may be used to propel a liquid that contains the surfactant:DNA complexes toward the surface of the individual's skin. The liquid is propelled at a sufficient velocity such that upon impact with the skin the liquid penetrates the surface of the skin, permeates the skin and muscle tissue therebeneath. In some embodiments, a needleless injection device may be used to deliver compositions of this invention to tissue of other organs in order to introduce a nucleic acid molecule to cells of that organ.

According to methods of this invention, complexes or compositions of this invention may be administered directly into the individual to be immunized. By any route, the compositions of this invention are introduced into cells which are present in the body of the individual. Delivery of the polynucleic acid sequences which encode target proteins can confer mucosal immunity in individuals immunized by a mode of administration in which the material is presented in tissues associated with mucosal immunity. Thus, in some examples, the complex of this invention is delivered by administration in the buccal cavity within the mouth of an individual, or administered rectally, vaginally, or to the urethra.

Alternatively, the compositions may be introduced by various means *ex vivo* into removed cells of the individual which are reimplanted after administration. Such means include, for example, *ex vivo* transfection, electroporation, microinjection and microprojectile bombardment. After the complex of the invention is taken up by the cells, the cells are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have the polynucleic acid sequences incorporated therein can be implanted into the individual even if the vaccinated cells were originally taken from another individual.

In some embodiments, the compositions of the present invention comprise as the polynucleic acid sequence an attenuated viral vaccine that may be delivered as a genetic construct. Such constructs may allow for production of viral particles. Delivery of the attenuated vaccine as a polynucleic acid sequence in a complex of this invention allows for an easier way to produce large quantities of safe, pure active immunizing product.

In some embodiments, the compositions of the present invention may be administered with or without the use microprojectiles. In some embodiments, the compositions of the present invention may be delivered to the cells of an individual free of solid particles. As used herein, the phrase "free of solid particles" is meant to refer to a liquid that does not contain any solid microprojectile used as a means to perforate, puncture or otherwise pierce the cell membrane of a cell in order to create a port of entry for compositions of this invention into the cell. For example, the compositions of the present invention are administered by means of a microprojectile particle bombardment procedure as taught by Sanford et al. in U.S Patent 4,945,050 issued July 31, 1990. In some embodiments of the invention, the compositions of the present invention are administered as part of a liposome complex.

The methods of the present invention are useful in the fields of both human and veterinary medicine. Accordingly, the present invention relates to genetic immunization of mammals, and vertebrates, such as birds and fish. The methods of the present invention can be particularly useful for mammalian species including human, bovine, ovine, porcine, equine, canine and feline species.

The following examples illustrate the preferred methods for preparing the benzylammonium-containing surfactant/polynucleic acid complexes of the present invention and further illustrate that such compositions facilitate the uptake of the polynucleic acid. These examples which employ as the surfactant, benzalkonium chloride or benzethonium chloride, and as the polynucleic acid sequences, plasmid sequences containing a herpes simplex virus gD gene merely illustrate selections of the surfactant, the polynucleic acid sequence, the type of sequence and source of the sequence. It is understood by one of skill in the art, that other selections for these components of the invention may be readily selected as taught by this specification. These examples are illustrative only and do not limit the scope of the invention.

EXAMPLE 1: A Benzylammonium-Containing Surfactant/Polynucleic Acid Formulation of The Invention

A composition containing uniform ionic, vesicular complexes with polynucleic acid packaged in an aqueous benzylammonium-containing surfactant is formulated according to this invention as follows. For this example, the polynucleic acid sequence component is plasmid DNA, and the benzylammonium-containing surfactant, benzylammonium chloride. The buffer used is phosphate buffer. Other conventional buffers, such as citrate buffer, also can be used instead of phosphate buffer. Tonicity of the resulting solution may be adjusted with sodium chloride, sucrose, other conventionally known isotonic agents, such as mannitol, sorbitol, trehalose, or any non-ionic agents from the list in *Remington's Pharmaceutical Sciences*, supra.

An illustrative formulation of this invention is prepared by admixing under conditions of ambient temperature the components listed in Table I. Generally, a stock solution of the benzylammonium-containing compound is prepared in the selected buffer buffer. A polynucleic acid solution, e.g., a DNA or RNA solution is prepared containing the selected concentration of polynucleic acid sequence, e.g, plasmid DNA, in the selected buffer with the tonicity adjusting agent. Before admixture, both solutions are preferably filtered conventionally, for example, using a

0.22 μm Millex GV syringe filter. Suitable amount of the surfactant solution is added to suitable volumes of the polynucleic acid by slow mixing. The desired concentration is made by selecting the concentration of surfactant solution and a desired concentration of the polynucleic acid solution.

A composition according to this invention must be soluble. The endpoint of concentrations of the components of the complex is generally precipitation. It is preferred that the charge ratio of the benzylammonium-containing surfactant and the polynucleic acid be less than 1, and that excess positive charge be avoided. The conditions of the solution, and the amounts of polynucleic acid sequence and surfactant can be manipulated to increase solubility and reduce the toxicity of the surfactant concentrations. Desirably, the pH of the admixture is between 6 and 8, and more preferably between 6.2 and 7.2. The desired isotonicity, hypotonicity (<0.9% w/v NaCl or equivalent) or hypertonicity (>0.9% w/v NaCl or equivalent), can be adjusted by tonicity adjusting agents [see, e.g., *Remington's*, cited above].

Table I provides an illustrative composition of the invention. Other ranges of the components of the soluble ionic complex of the invention are disclosed above.

Table I

Component	Range
Plasmid DNA	10μg/ml - 20.0 mg/ml
Benzylammonium containing surfactant	0.001 - 2.4% w/v
Buffer (e.g. phosphate)	2 - 30 mM
Sodium chloride	0 - 0.9% w/v
Tonicity adjusting agent (e.g., sucrose)	0 - 13% w/v

EXAMPLE 2: Scanning and Transmission Electron Microscopic Studies with Benzalkonium Chloride:DNA Complexes

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were employed to visualize the resulting complexes formed by the benzylammonium-containing surfactant and polynucleic acid compositions of this invention.

A. Preliminary Studies

Electron microscope studies were made of the following benzalkonium chloride/DNA complex formulations of the invention:

a) 0.025% benzalkonium chloride/0.5 mg/mL of DNA,

b) 0.0125% benzalkonium chloride/0.5 mg/mL DNA,

c) 0.025% benzalkonium chloride/0.1 mg/mL of DNA,

d) 0.012 % benzalkonium chloride/0.05 mg/mL of DNA. These formulations were made in the same manner as described for Example 1, except that the benzalkonium chloride concentrations are different as stated above. Each formulation containing 8.71% sucrose w/v and 5 mM phosphate buffer at pH6.7 ±0.5.

Electron microscopic photographs (not pictured) illustrated that each formulation formed uniform vesicular complexes indistinguishable from those derived from classical liposomes and cationic liposomes.

B. Additional SEM and TEM Studies

i. Formulations of this invention were prepared by mixing DNA at 0.5 mg/mL, with benzalkonium chloride (0.02% w/v) in 10 mM citrate or phosphate buffers, pH 6.7 (± 0.5), and 50 mM NaCl, substantially as described above.

The structures visible by both SEM and TEM are indistinguishable from those derived from classical liposomes and cationic liposomes. The size of these particles varied from 50 nm to 230 nm. However, most structures had an uniform size distribution ranging from 70 to 100 nm.

ii. Additional studies were performed by mixing DNA at 5 mg/ml in higher concentrations of benzalkonium chloride, up to 0.04% w/v, in 10 mM citrate or phosphate buffers, pH 6.7 (± 0.5), and 50 mM NaCl, substantially as described

above. Benzalkonium chloride alone does not form vesicular complexes in water. At higher than ≥0.1% w/v, benzalkonium chloride alone is soluble and forms micelles. However, upon addition of DNA, vesicular complexes are formed, when observed by SEM. The vesicular complexes range in size from 50 (at 0.01% benzalkonium chloride) to 400 nm particles (0.04% w/v benzalkonium chloride). Benzalkonium chloride at concentrations higher than ≥ 0.04% w/v under these conditions, in the presence of DNA formed a fine precipitate that contained DNA (determined by agarose gel electrophoresis of the precipitate). At concentrations above 0.04% benzalkonium chloride under these conditions, the precipitates were snowy and flocculent.

These observations and the SEM and TEM pictures described herein (not pictured) show that benzalkonium chloride:DNA compositions according to this invention form a vesicular-like or liposomal-like structure. The quaternary ammonium cationic head group of the benzalkonium chloride is positively charged independent of protonation, and forms complexes with DNA, that decrease its solubility. At benzalkonium chloride concentrations lower than 0.04% w/v in the compositions above, the complexes remain soluble, while at benzalkonium chloride concentrations ≥0.04% w/v in the compositions above, the hydrophilicity of these complexes is reduced to near neutrality. The association of DNA with the precipitates further indicates complexation.

iii. SEM was carried out on formulations that contained two different concentrations of DNA (100 μg/ml and 0.5 mg/ml), and fixed concentrations of benzalkonium chloride (0.02% w/v). Similar particle distribution was found at both concentrations of DNA, but in larger numbers with higher DNA concentrations. Larger particles in higher DNA concentrations appear to have derived from the fusion of several 50 nm particles. Formation of larger particles was dependent on the concentration of DNA in the formulation. These particles were visualized by shadow casting in SEM analysis using carbon, and were visible by short (10 second) exposure to the contrasting agent uranyl acetate in TEM analysis. The TEMs show membranous structures typical of liposomes described in literature.

As controls, SEM of DNA alone and benzalkonium chloride alone in aqueous solutions were performed. Vesicular structures are found only when aqueous solutions of DNA and benzalkonium chloride are mixed. TEMs show structures that are vesicular and some are multilamellar. Membranous structures found in these TEMs are consistent with the formation of lamellar vesicular complexes in liposomes. These results show that benzalkonium chloride:DNA complexes form lamellar and vesicular structures, similar to those described for liposomes and liposomal formulations.

EXAMPLE 3: Labeled DNA is Associated with the Vesicular complexes, SEM and TEM Analysis

A. Preparation of Open Circular Plasmid DNA.

Supercoiled (SC) plasmid DNA was converted into an open-circular form by heating at 80°C, for 4 hours. Nearly 80% of SC DNA was converted to nicked open circular (OC) form by this method. The amount of DNA converted to OC was quantified using a video gel-scanner, following electrophoretic separation of SC and OC forms. The OC was purified to nearly 95% purity on a Q-Sepharose matrix using a NaCl step gradient.

B. Preparation of Biotinylated dUTP DNA.

Fifty micrograms of nicked OC DNA was subjected to a strand displacement reaction using Klenow (the non-proofreading proteolytic fragment of DNA polymerase of *E. coli*), and dNTP. The dNTP mixture contained dATP, dGTP, dCTP, dUTP, and Bio-dUTP. Bio-dUTP has biotin on an 11 carbon linker attached to the base uracil. The nucleotide concentration was at 50 μM, while the bio-dUTP was at 2 μm, and dUTP was at 20 μM. The plasmid was purified from free nucleotides by two rounds of ethanol precipitation, and two 70% ethanol washes. The amount of biotin incorporated into the plasmid was determined by a kinetic ELISA, by reacting dilutions of the plasmid with streptavidin-horseradish peroxidase (HRP). Three molecules of biotin were incorporated per plasmid molecule.

C. Preparation of Streptavidin-Colloidal Gold Conjugated Plasmid DNA.

One microgram of a 5 Kbp plasmid DNA corresponds to 1.25×10^{14} molecules (3.75×10^{13} biotin). Five hundred micrograms of mixture in one milliliter was reacted with 10^{11} molecules of gold-conjugated streptavidin. This ensures absence of free streptavidin-gold conjugates [T. Daemen et al, Hepatology, 26:416 (1997)]. Benzalkonium chloride at 0.02% was added to the mixture to form complexes. The mixture was analyzed by TEM.

D. TEM Analysis

TEM analysis of the above-prepared gold labeled DNA in the benzylammonium-containing surfactant/polynucleic acid sequence compositions of this invention demonstrated that the gold-labeled DNA is found in structures (complexes) that are identical to those found by carbon shadowing. These structures show that DNA is within the vesicular membranes. Vesicular complexes in some fields appear to be multilamellar, and the gold labeled plasmid molecules are interspersed within these lamellae. Electron diffraction shows densities corresponding to gold that were detected on these membranes, and within membranes. This analysis demonstrates that benzalkonium chloride:DNA complexes form vesicular complexes, and the DNA is in the vesicular space, intimately associated with the membrane.

EXAMPLE 4: Enhancement of DNA Uptake Using Compositions of the Invention

A. The Formulations Tested

Four different formulations were prepared to evaluate the ability of aqueous mixtures of benzylammonium-containing surfactants and polynucleic acid sequences to facilitate DNA delivery. The four formulations of the invention and the control are reported in Table 2 below.

Formulation 1 (2 mL formulation) was prepared as follows: 0.5% benzalkonium chloride stock was prepared in 5 mM phosphate buffer. A DNA plasmid was constructed which contained the Herpes Simplex Virus gene encoding the gD_2 protein linked to a cytomegalovirus promoter and SV40 polyadenylation site. This plasmid, referred to as plasmid 24, is described in detail in International Patent

Publication No. WO97/41892, published November 13, 1997. The DNA plasmid solution was prepared containing 0.5 mg/mL plasmid DNA in 5 mM phosphate buffer with 8.71% sucrose. Both the benzalkonium chloride stock solution and DNA solution were filtered using 0.22 μm Millex GV syringe filter. Forty μL of benzalkonium chloride stock solution was added to 1.96 mL of DNA solution (0.5 mg/mL) by slow mixing.

Formulation 2 (2 mL formulation) was prepared as follows: 0.5% benzalkonium chloride stock was prepared in 5 mM phosphate buffer. DNA solution contain the above-described plasmid 24 (0.5 mg/mL) was prepared in 5 mM phosphate buffer with 8.71% sucrose. Both the benzalkonium chloride stock solution and DNA solution were filtered using 0.22 μm Millex GV syringe filter. Eighty μL of benzalkonium chloride stock solution was added to 1.92 mL of DNA solution (0.5 mg/mL) by slow mixing.

Formulation 3 (2mL formulation) was prepared as follows: 0.5% benzalkonium chloride stock was prepared in 5 mM phosphate buffer. DNA solution containing the above-described plasmid 24 (0.5mg/mL) was prepared in buffer solution containing 5 mM phosphate and 150 mM of sodium chloride. Both the benzalkonium chloride stock solution and DNA solution were filtered using 0.22 μm Millex GV syringe filter. One hundred twenty μL of benzalkonium chloride stock solution was added to 1.88 mL of DNA solution (0.5 mg/mL) by slow mixing.

As a positive control, a DNA only formulation was prepared containing 0.5 mg/mL DNA of plasmid 24 in 30 mM citrate buffer, 0.1% ethylenediamine tetraacetic acid (EDTA), 150 mM NaCl at pH 6.7±0.5. As a negative control, a DNA only formulation was prepared containing 0.5 mg/ml DNA of plasmid 23 (no HSV insert) in 30 mM citrate buffer, 0.1% EDTA, 150 mM NaCl at pH 6.7±0.5.

Table 2

Components	Formulations		
	1	2	3
Plasmid DNA (mg/mL)	0.5	0.5	0.5
Benzalkonium chloride (w/v)	0.01%	0.02%	0.06%
Sucrose (w/v)	8.71%	8.71%	0
Sodium Chloride (mM)	0	0	150
Phosphate buffer (mM)	5	5	5
pH	6.7±0.5	6.7±0.5	6.7±0.5

B. Protocol of Test

Six groups of BALB/c mice (5 mice/group) were immunized intramuscularly with 50 μg plasmid DNA in a total volume of 100 μL per dose, which was distributed between 3-4 sites per leg on Day 1. The plasmid was administered in compositions with different concentrations of benzalkonium chloride as follows:

Group I received Formulation 1 (50 μg plasmid in 0.01% benzalkonium chloride). Group II received Formulation 2 (50 μg plasmid in 0.02% benzalkonium chloride). Group III received Formulation 3 (50 μg plasmid in 0.06% benzalkonium chloride). Group IV received positive control (50 μg plasmid 24 without any transfection facilitating agent); and Group V received negative control (plasmid 23 with no HSV insert and without any transfection facilitating agent).

Serum was collected prior to first injection (Day 0) and at two weeks post injection (Day 14). Animals were boosted once more at the same dosage at four weeks (Day 28). After the final injection an additional serum sample was taken. Two weeks following the final immunization (Day 42), mice were euthanized using halothane, followed by cervical dislocation and the spleens were harvested.

C. In Vivo Results

The sera and spleens from each mouse and each group of mice was assayed for humoral response, i.e., antibody response to gD, as measured by a standard enzyme linked immunosorbent assay (ELISA) [J. E. Coligan et al, eds., "Current Protocols in Immunology", Vol. 1, chap. 2.1, John Wiley & Sons, Inc. (1992)]. Figs. 1 and 2 illustrate the humoral responses of individual animals and the group average humoral responses, respectively. Antibody response calculations were conducted as follows. Based on linear regression, a linear model [O.D.= (slope x antibody concentration) + Intercept] was fitted to the standard data. The equation for the best fit.line was used to calculate antibody response for specific formulations. Fig. 3 illustrates the individual and group cellular responses of the same animals. Systemic cellular response (SI) was measured using a splenic cell proliferation assay in which was calculated the ratio of the number of spleen cells stimulated in the presence of HSV gD antigen and radiolabelled nucleotides divided by the number of the same spleen cells incubated in the absence of any antigen, but in the presence of radiolabelled nucleotides [J. E. Coligan et al, eds., "Current Protocols in Immunology", Vol. 1, chap. 2.1, John Wiley & Sons, Inc. (1992)].

Fig. 1 demonstrates that Formulation 1 (0.01% benzalkonium chloride and 0.5 mg/mL DNA) produced a significantly higher individual and group humoral response than did the positive control DNA. The group average humoral response (antibody) for Formulation 1 is 2.9 ng/mL higher than the positive control DNA alone formulation. Fig. 1 demonstrates that all five animals in Groups I and II gave humoral responses to Formulations 1 and 2, respectively; whereas only four animals in Group IV, which received the positive control DNA, produced humoral responses. The negative control DNA gave no response, as predictable. Thus, greater consistency and predictability of immune responses was seen with formulations of this invention than with DNA delivered in the absence of a transfection facilitating agent of this invention. Formulation 3 in the preparation used in this experiment produced an undesirable precipitate.

Fig. 3 demonstrates that the cellular responses for Formulations 1 and 2 (Groups I and II, respectively) are comparable to those elicited by administration of positive control DNA only (Group IV). However, again, variation in the cellular responses to formulations of this invention is minimal, compared to the wide variation in responses observed for those animals in Group IV that received DNA alone.

Based on the humoral and cellular responses in animals, as described above, it appears that benzalkonium chloride/DNA complex formulations (Formulations 1 and 2) provide better immune responses compared to DNA alone and facilitates the transfer of DNA into the subject.

EXAMPLE 5: Benzethonium Chloride:DNA Complexes

Compositions according to the present invention were also prepared using another exemplary benzylammonium-containing surfactant as described here, i.e., benzethonium chloride. These formulations were prepared in substantially the same manner as described for the benzalkonium chloride formulations of Example 4 above. The different formulations appear in Table 3 below.

Table 3

Components	Formulation 1	Formulation 2	Formulation 3
Plasmid DNA (mg/mL)	0.1	0.5	0.5
Benzethonium chloride (w/v)	0.01%	0.01%	0.02%
Sucrose (w/v)	8.71%	8.71%	8.71%
Phosphate buffer (mM)	5	5	5
pH	6.7±0.5	6.7±0.5	6.7±0.5

TEM and SEM analyses of these compositions were performed as described in Example 3. Based on TEM and SEM analysis, the structures have the same description as found for the benzalkonium chloride:DNA vesicular complexes of Example 3, i.e. uniformly sized vesicular complexes which packaged DNA.

All above-noted published references, and the provisional United States patent application No. 60/063,360, are incorporated herein by reference. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

What is claimed is:

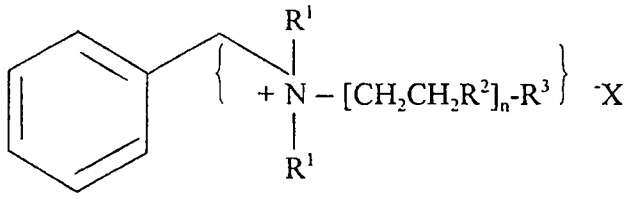
1. A soluble ionic complex comprising an aqueous mixture of

(a) a benzylammonium group-containing surfactant; and

(b) a polynucleic acid sequence.

2. The complex according to claim 1, which forms a vesicular complex containing said polynucleic acid sequence substantially packaged therein.

3. The complex according to claim 1, wherein said benzylammonium group-containing surfactant is a surfactant of the formula:



wherein X is an anion;

each R^1 is independently selected from the group consisting of a hydrogen and a lower alkyl group comprising from 1 to 6 carbon atoms;

R^2 is selected from the group consisting of CH_2 and -O-;

R^3 is selected from the group consisting of H, CH_3, C_2H_5, phenyl, mono-substituted phenyl, and di-substituted phenyl, wherein said substitutions are independently selected from the group consisting of C_1-C_{10} branched or straight chain alkyls groups; and

n is an integer of 2 through 7, provided that when n is 1, R^3 is methyl, ethyl, phenyl or substituted phenyl; when n is 4 through 6, R^3 is H, methyl, ethyl or phenyl; when n is 6, R^3 is H, methyl or ethyl; and when n is 7, R^3 is H or methyl.

4. The complex according to claim 3, wherein X is selected from the group consisting of halide, sulfate, and carbonate.

5. The complex according to claim 4, wherein said benzylammonium group-containing surfactant is a benzalkonium halide.

6. The complex according to claim 5 wherein said halide is chloride and said surfactant is benzalkonium chloride.

7. The complex according to claim 3, wherein said surfactant is a benzethonium halide.

8. The complex according to claim 7, wherein said surfactant is benzethonium chloride.

9. The complex according to claim 1, wherein said benzylammonium containing surfactant is present in a concentration of about 0.001 to about 2.4% w/v.

10. The complex according to claim 9, wherein said benzylammonium containing surfactant is present in a concentration of about 0.001 to about 0.1% w/v.

11. The complex according to claim 10, wherein said benzylammonium containing surfactant is present in a concentration of about 0.005 to about 0.06% w/v.

12. The complex according to claim 11, wherein said benzylammonium containing surfactant is present in a concentration of about 0.005 to about 0.03% w/v.

13. The complex according to claim 1, wherein said polynucleic acid sequence comprises a ribonucleic acid sequence.

14. The complex according to claim 1, wherein said polynucleic acid sequence comprises a dioxyribonucleic acid sequence.

15. The complex according to claim 14, wherein said deoxyribonucleic acid sequence is a plasmid.

16. The complex according to claim 15, wherein said plasmid comprises a nucleotide sequence that encodes a protein or peptide, said sequence operably linked to regulatory sequences directing expression of said protein or peptide in a host cell.

17. The complex according to claim 14, wherein said sequence encodes a protein or a peptide.

18. The complex according to claim 1 wherein said polynucleic acid sequence is present in said complex in a concentration of between about 10μg/ml to about 20 mg/ml.

19. The complex according to claim 18 wherein said polynucleic acid sequence is present in said complex in a concentration of between about 50μg/ml to about 10 mg/ml.

20. The complex according to claim 19 wherein said polynucleic acid sequence is present in said complex in a concentration of between about 100μg/ml to about 1.0 mg/ml.

21. The complex according to claim 1 which further comprises additives selected from the group consisting of buffering agents and tonicity adjusting agents.

22. The complex according to claim 1, wherein said surfactant is benzalkonium chloride, said polynucleic acid is plasmid DNA, and said aqueous mixture is an isotonic solution.

23. A composition comprising a polynucleic acid sequence substantially packaged in a vesicular complex formed by an aqueous mixture of a benzylammonium-containing surfactant and said sequence.

24. A composition comprising multiple vesicular complexes of uniform size, each said vesicular complex containing a polynucleic acid sequence substantially packaged in a vesicular complex formed by an aqueous mixture of a benzylammonium-containing surfactant and said sequence.

25. The composition according to claim 24 formed by mixing an aqueous solution of a benzylammonium-containing surfactant with a polynucleic acid sequence.

26. A pharmaceutical composition comprising the soluble ionic complex of any of claims 1 through 22 in a suitable pharmaceutical carrier.

27. The composition according to claim 26, which is an injectable formulation.

28. A pharmaceutical composition comprising the vesicular complex composition of claims 23 or a composition of any of claims 24 through 25 in a suitable pharmaceutical carrier.

29. A method of introducing a polynucleic acid sequence into a cell comprising the step of contacting said cell with a member of the group consisting of:

(a) a complex of any of claims 1 through 22;

(b) a composition of claim 23; and

(c) a composition of any of claims 24-25; and

(d) a composition of any of claims 26-29.

30. A method of facilitating the uptake of a polynucleic acid sequence into a cell comprising contacting said cell with a soluble ionic complex comprising an aqueous mixture of

(a) a benzylammonium group-containing surfactant; and

(b) a polynucleic acid sequence.

31. The method according to claim 30, wherein said complex is in the form of a vesicular complex in which the polynucleic acid sequence is substantially packaged.

32. A method of inducing an immune response in a mammalian subject to a pathogenic antigen comprising the step of administering to cells of said subject, an effective amount of a composition comprising the complex of claim 1 or 2, wherein said polynucleic acid sequence comprises a sequence which encodes at least one epitope that is identical or substantially similar to an epitope of a antigen of said pathogen, said epitope-encoding sequence under the control of regulatory sequences that direct expression of said protein in the cells of said subject.

33. The method according to claim 32 wherein composition is administered by a route selected from the group consisting of intramuscularly, intraperitoneally, intradermally, subcutaneously, intravenously, intraarterially, intraoccularly, orally, topically, transdermally, intrathecally, intranasally, rectally, vaginally, interurethally, buccally and by inhalation.

34. A method of immunizing a mammalian subject against a disease comprising the step of administering to said subject a composition comprising an effective amount of a composition comprising a complex of claim 1 or 2, wherein said polynucleic acid sequence comprises a nucleotide sequence encoding a target protein, said protein comprising an epitope identical or substantially similar to an epitope of a protein associated with cells that characterize said disease, said protein encoding sequence operatively linked to regulatory sequences directing the expression of said protein in the cells of said subject.

35. The method according to claim 36 wherein said nucleic acid molecule is administered by a route selected from the group consisting of intramuscularly, intraperitoneally, intradermally, subcutaneously, intravenously, intraarterially, intraoccularly, orally, topically, transdermally, intrathecally, intranasally, rectally, vaginally, interurethally, buccally and by inhalation.

36. A method of treating a mammalian subject for a disease comprising the step of administering to cells of said subject, an effective amount of a composition comprising a complex of claim 1 or 2, wherein said polynucleic acid sequence comprises a sequence which encodes a protein or peptide that produces a therapeutic effect on the subject, said protein-encoding sequence under the control of regulatory sequences that direct expression of said protein in the cells of said subject.

37. The method according to claim 36, wherein said nucleic acid molecule is administered by a route selected from the group consisting of intramuscularly, intraperitoneally, intradermally, subcutaneously, intravenously, intraarterially, intraoccularly, orally, topically, transdermally, intrathecally, intranasally, rectally, vaginally, interurethally, buccally and by inhalation.

38. A method of treating a mammalian subject for a disease comprising the step of administering to cells of said subject, an effective amount of a composition comprising a complex of claim 1 or 2, wherein said polynucleic acid sequence comprises a sequence which encodes a protein that compensates for a missing, non-functional or partially functioning native mammalian protein, said protein-encoding sequence under the control of regulatory sequences that direct expression of said protein in the cells of said subject.

39. The method according to claim 38 wherein said nucleic acid molecule is administered by a route selected from the group consisting of intramuscularly, intraperitoneally, intradermally, subcutaneously, intravenously, intraarterially, intraoccularly, orally, topically, transdermally, intrathecally, intranasally, rectally, vaginally, interurethally and buccally. and by inhalation.

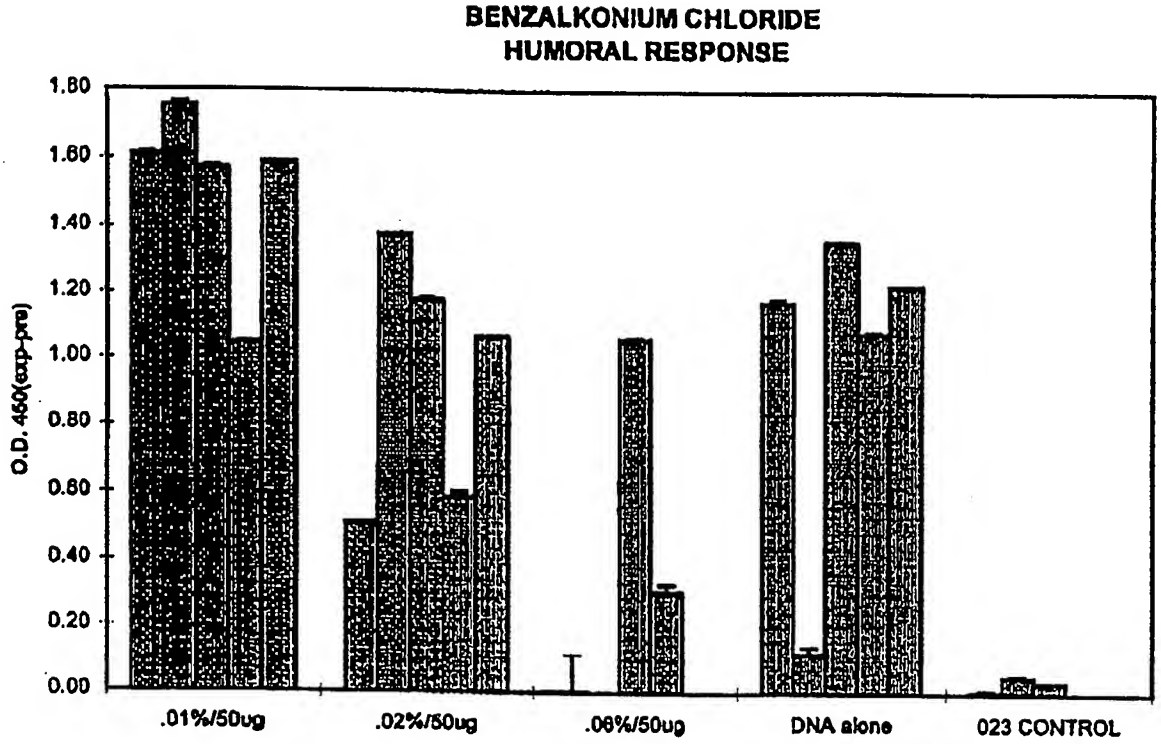

BENZALKONIUM CHLORIDE
HUMORAL RESPONSE
O.D. 460(exp-pre)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
.01%/50ug
.02%/50ug
.06%/50ug
DNA alone
023 CONTROL

FIG. 1

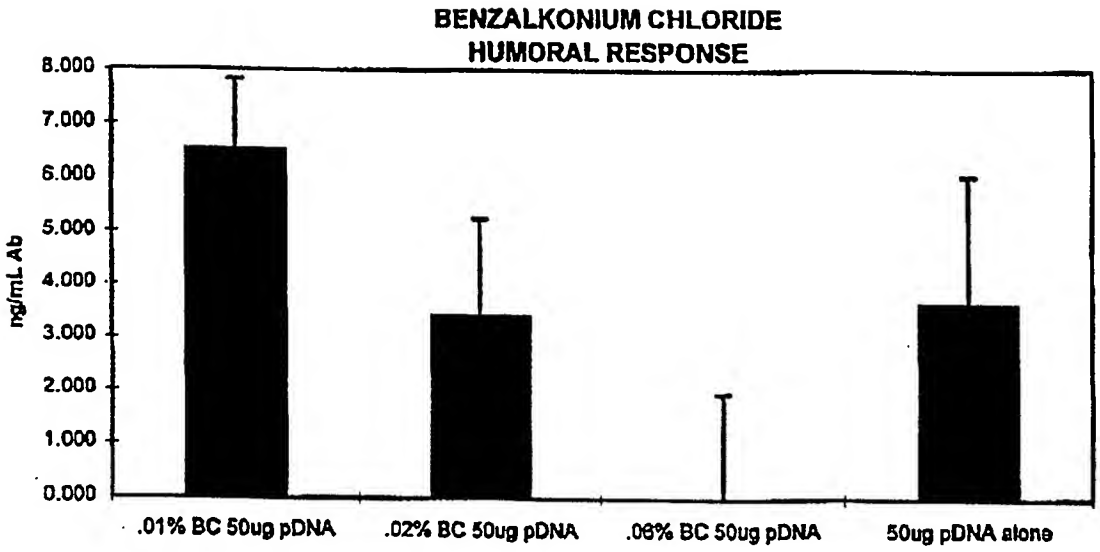

BENZALKONIUM CHLORIDE
HUMORAL RESPONSE
ng/mL Ab
8.000
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
.01% BC 50ug pDNA
.02% BC 50ug pDNA
.06% BC 50ug pDNA
50ug pDNA alone

FIG. 2

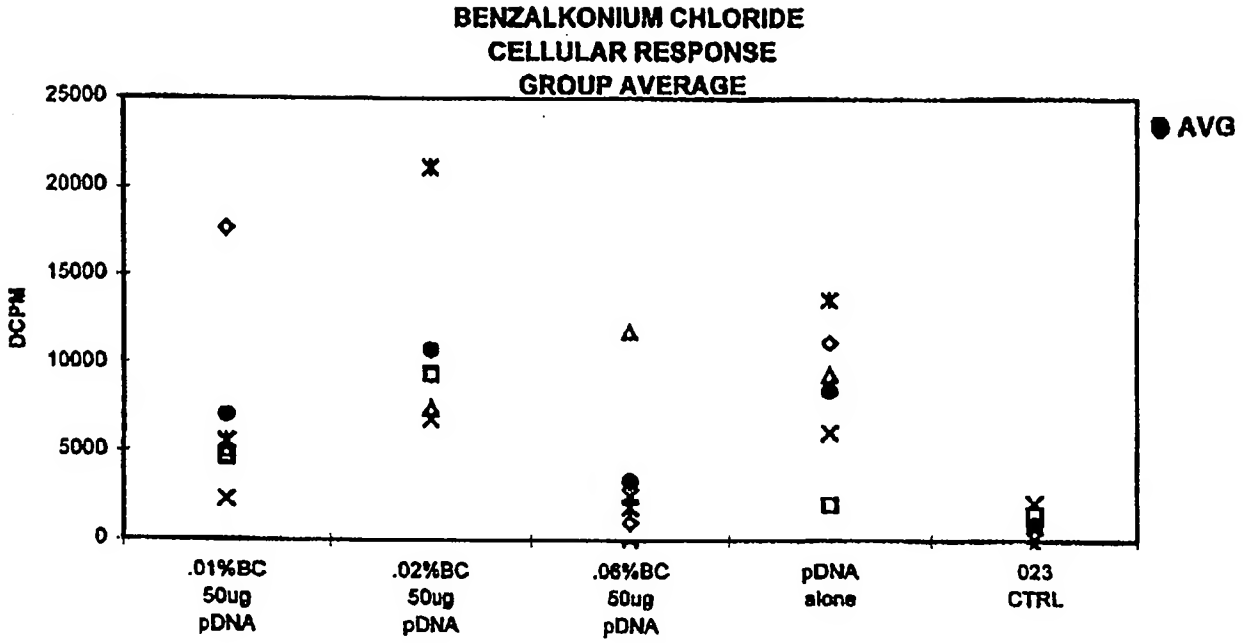

BENZALKONIUM CHLORIDE
CELLULAR RESPONSE
GROUP AVERAGE
DCPM
25000
20000
15000
10000
5000
0
AVG
.01%BC 50ug pDNA
.02%BC 50ug pDNA
.06%BC 50ug pDNA
pDNA alone
023 CTRL

FIG. 3

Characterization and Biological Evaluation of a Microparticle Adjuvant Formulation for Plasmid DNA Vaccines

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ABSTRACT: We describe the physiochemical characterization and immunological evaluation of plasmid DNA vaccine formulations containing a nonionic triblock copolymer adjuvant (CRL1005) in the presence and absence of a cationic surfactant, benzalkonium chloride (BAK). CRL1005 forms particles of 1–10 microns upon warming above its phase-transition temperature (~6–8°C) and the physical properties of the particles are altered by BAK. DNA/CRL1005 vaccines formulated with and without BAK were evaluated in rhesus macaques to determine the effect of CRL1005 and BAK on the ability of plasmid DNA to induce a cellular immune response. Immunogenicity results indicate that the addition of CRL1005 to human immunodeficiency virus-1 *gag* plasmid DNA formulated in phosphate-buffered saline leads to an enhancement in the gag-specific cellular immune response. Moreover, the addition of BAK to human immunodeficiency virus-1 *gag* plasmid DNA/CRL1005 formulations produces an additional enhancement in gag-specific cellular immunity. *In vitro* characterization studies of DNA/CRL1005 formulations indicate no detectable binding of DNA to CRL1005 particles in the absence of BAK, suggesting that the enhancement of cellular immunity induced by DNA/CRL1005 formulations is not due to enhanced DNA delivery. In the presence of BAK, however, results indicate that BAK binds to CRL1005 particles, producing cationic microparticles that bind DNA through electrostatic interactions. If BAK is present at the phase-transition temperature, it reduces the particle size from ~2 microns to ~300 nm, presumably by binding to hydrophobic surfaces during particle formation. Zeta potential measurements indicate that the surface charge of CRL1005-BAK particles changes from positive to negative upon DNA binding, and DNA bound to the surface of CRL1005-BAK particles was visualized by fluorescence microscopy. These results indicate that the addition of BAK to DNA/CRL1005 formulations leads to the formation of ~300 nm CRL1005-BAK-DNA particles that enhance the cellular immune response in rhesus monkeys. J Pharm Sci 93:1924–1939, 2004

Keywords: DNA; DNA delivery; vaccine adjuvants; microparticles; particle sizing; light-scattering; immunology

INTRODUCTION

Plasmid DNA-based vaccines have the potential for significant advances beyond both live virus and protein-based vaccines in terms of stability,

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safety and characterizability. However, large doses of DNA are required to induce sufficiently robust immune responses in humans and nonhuman primates.[1–4] Therefore, a need exists for the identification of safe and effective adjuvants to enhance the ability of plasmid DNA-based vaccines to stimulate both humoral and cellular immunity.

CRL1005 is a nonionic triblock copolymer composed of blocks of polyoxypropylene (POP) and polyoxyethylene (POE).[5] Linear triblock POE-POP-POE copolymers are referred to by the BASF trade name Pluronic® or by the term *poloxamer* and were originally developed by Wyandotte Chemicals Corporation and BASF Performance in the early 1950s, for use as surfactants.[5] The early copolymers had POP cores up to ~4,000 Da (from 16 to 67 POP blocks and from 2 to 122 POE blocks). The CRL series copolymers differ from the older Pluronic® copolymers by having a much larger molecular weight POP core (9–20 kDa POP core containing 155–345 POP blocks, and with 2.5–20% POE). CRL1005 has a POE content of 5% and a POP core of 12,000 Da. The larger molecular weight of the CRL copolymers endows them with unusual solubility properties. Typically, these copolymers are soluble in aqueous solutions at near ice temperatures but they aggregate into microparticles above their phase-transition (cloud-point) temperature. It is believed that the adjuvant properties of the CRL copolymers are related to their ability to aggregate into surface-active particles.[6,7]

Plasmid DNA vaccines formulated with CRL1005 have been shown to significantly enhance the levels of antigen-specific cellular immune responses in rhesus monkeys.[8] Moreover, the results indicate that a DNA/CRL1005 formulation containing benzalkonium chloride (BAK) induced even higher levels of $CD8^+$ gag-specific T cells than the DNA/CRL1005 formulation without BAK, during the priming series of three vaccinations.[9] These results suggest that the combination of CRL1005 and BAK is an effective adjuvant for plasmid DNA vaccines in rhesus monkeys.

We have characterized DNA/CRL1005 formulations in the presence and absence of BAK to determine how the physical properties of the formulation are altered by the presence of this cationic surfactant. We believe that understanding the effects of BAK on the DNA/CRL1005 formulation might suggest possible mechanisms of immunogenicity enhancement and guide the direction of future formulation development. Our results indicate that BAK binds to CRL1005 particles through hydrophobic interactions, producing cationic microparticles that subsequently bind DNA through electrostatic interactions.

In this report, we describe the effects of BAK on the size and surface charge of CRL1005 particles and show that DNA-BAK precipitates do not exist in these DNA/CRL1005/BAK formulations above the cloud point of CRL1005. We also demonstrate that other cationic surfactants may be used in place of BAK to enhance the binding of plasmid DNA to CRL1005 particles. These results show that CRL1005/cationic surfactant formulation composition and preparation methods can be easily manipulated to alter the particle size, charge, and amount of DNA bound to the surface, suggesting that this system may be useful to explore the effects of many different variables on the adjuvant properties of CRL1005.

EXPERIMENTAL

Materials

CRL1005 (TranzFect) was obtained from CytRx Corporation (Atlanta, GA). BAK (a mixture of several homologs of differing chain length) was purchased from Spectrum (New Brunswick, NJ) or from Ruger Chemical Co. (Irvington, NJ). Dodecyl trimethylammonium bromide (BAK-12), tetradecyl trimethylammonium bromide (BAK-14), and hexadecyl trimethylammonium bromide (BAK-16) were purchased from Sigma Chemical Co. (St. Louis, MO) for use as high-performance liquid chromatography (HPLC) reference standards. Cetyl pyridinium chloride (CPC) was obtained from Zeeland Chemical (Zeeland, Michigan) or from Spectrum. Cetyl trimethylammonium chloride (CTAC) was obtained from Spectrum and PicoGreen was purchased from Molecular Probes (Eugene, OR).

Vaccine Vector

A synthetic gene for *gag* from a Clade B sequence human immunodeficiency virus (HIV)-1 was previously constructed using codons frequently used in humans.[10,11] The *gag* gene was inserted into the V1Jns plasmid (V1Jns-gag) under the control of the human cytomegalovirus/human intron A promoter and bovine growth hormone terminator.[12,13]

Description of Formulations

The formulations used in the rhesus immunogenicity study and for most of the *in vitro* characterization studies were (1) D101; 5 mg/mL V1Jns-gag plasmid DNA in phosphate-buffered saline (PBS), (2) D113; 5 mg/mL V1Jns-gag plasmid DNA in PBS containing 7.5 mg/mL CRL1005, and (3) D118; 5 mg/mL V1Jns-gag plasmid DNA in PBS containing 7.5 mg/mL CRL1005 and 0.5–0.6 mM BAK.

Preparation, Handling, and Storage of Formulations Containing CRL1005

Plasmid DNA formulations containing CRL1005 were prepared by adding CRL1005 to an aqueous solution of plasmid DNA in PBS at room temperature, followed by the addition of BAK. Care was taken to ensure complete solubilization of the CRL1005 by performing several cycles of cooling on ice, vigorous vortexing, then warming above the phase-transition temperature with additional vortexing. Care was also taken to ensure complete solubilization of the CRL1005, by incubation on ice, just before −70°C storage. Before use for either immunization studies or the *in vitro* characterization studies, CRL1005-containing formulations were removed from −70°C storage then warmed above the cloud point by incubation at room temperature. Rapid thawing of the frozen CRL1005 formulations in a water bath was avoided because this causes a significant reduction in the particle size, particularly in formulations lacking BAK.

Cloud-Point Determinations

The cloud-point temperature of the D118 formulation was determined using a model 62DS Aviv Circular Dichroism Spectrometer. A temperature scan from −1 to 14°C was performed while recording the output CD-Dynode signal at a wavelength of 360 nm, to monitor the light-scattering intensity. The onset of particle formation (i.e., the temperature at which the CD-Dynode signal begins to increase above the baseline) was defined as the cloud point of the formulation. The cloud point of the D118 formulation by this method (6°C) is specific for this particular formulation and therefore does not correspond to the lower critical solution temperature of the CRL1005 polymer.

Immunization

Rhesus macaques were between 3–10 kg in weight. In all cases, the total vaccine dose was suspended in 1 mL of PBS. The macaques were anesthetized (ketamine/xylazine) and the vaccines were delivered intramuscularly (im) in 0.5-mL aliquots into both deltoid muscles using tuberculin syringes (Becton-Dickinson, Franklin Lakes, NJ). Sera and peripheral blood mononuclear cells (PBMCs) were prepared from blood samples collected at several time points during the immunization regimen. All animal care and treatment were in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council.

IFN-γ ELISPOT Assay

Ninety-six-well flat-bottomed plates (Immobilon-P membrane; Millipore) were coated with 1 μg/well of anti-gamma interferon (IFN-γ) MAb MD-1 (U-Cytech-BV) overnight at 4°C. The plates were then washed three times with R10 medium [RPMI (Gibco-BRL), 10% fetal bovine serum (HyClone), 50 μM 2-mercaptoethanol (Gibco-BRL), 1 mM HEPES (Gibco-BRL), 200 μM L-glutamine, 100 μM sodium pyruvate, penicillin-streptomycin (Gibco-BRL)] and then the plates were blocked in R10 for 2 h at 37°C. The solution was discarded from the plates, and freshly isolated PBMCs were added at $2–4 \times 10^5$ cells/well. The cells were stimulated in the absence (mock) or presence of the HIV-1 *gag* peptide pool (4 μg/mL per peptide) or concanavalin A (Sigma) per milliliter. Cells were then incubated for 20–24 h at 37°C in 5% CO_2. Plates were washed six times with PBST [PBS (Gibco-BRL) containing 0.05% Tween 20 (Sigma)], and 1 μg of rabbit anti-IFN-γ polyclonal biotinylated detector antibody solution (U-Cytech-BV) per well was added, and the plates were incubated overnight at 4°C. The plates were washed six times with PBST and 1:2500 dilution of streptavidin-alkaline phosphatase conjugate (Pharmingen) was added per well. Color was developed by incubating in NBT/BCP (Nitro-blue tetrazolium chloride/5-Bromo-4-chloro-3′-indolyphosphate p-toluidine salt) (Pierce) for 10 min. Spots were counted under a dissecting microscope and normalized to 1×10^6 PBMCs.

Static Laser Diffraction Light Scattering

Particle-size distributions were generated by static laser diffraction light-scattering analysis of samples at room temperature using a Mastersizer 2000 (Malvern Instruments). Because of the high turbidity of the CRL1005 formulations, samples were diluted ~100-fold with degassed and 0.22-μm filtered PBS before the measurement. Degassed PBS was used for a background control measurement. Care was taken to use gentle mixing of diluted samples to avoid the generation of bubbles. A refractive index of 1.45 was used for the light-scattering measurements of CRL1005-containing formulations.

Zeta Potential and Dynamic Light Scattering

Zeta potential measurements and particle-size analyses by dynamic light scattering, shown in Table 1, were determined using a Zetasizer 3000 (Malvern Instruments). All samples were diluted 46-fold with 20 mM Tris-acetate (pH 7.2) before zeta potential measurements. The particle-size measurements of samples containing CRL1005 were performed after diluting samples by 50-fold with PBS. The particle size of plasmid DNA was determined at two concentrations, 0.2 and 2.5 mg/mL plasmid DNA in PBS. Data were collected for five 20-s cycles for each sample then averaged to yield the final value. As a control, a -50 ± 5 mV zeta potential standard (Malvern Instruments) was used.

Table 1. Surface Charge (Zeta Potential) and Hydrodynamic Diameter (Dh) of CRL1005 Formulations

Formulation[a]	Zeta Potential (mV)	Dh (nm)
DNA	−48.5	66, 62[b]
CRL1005	0.9	2178
CRL1005 + DNA	2.7	2468
CRL1005 + BAK	10.2	225
CRL1005 + BAK + DNA	−46.7	330

[a]The concentrations of DNA, CRL1005, and BAK were 5 mg/mL, 7.5 mg/mL, and 0.6 mM, respectively. Measurements were taken on CRL1005 samples after vigorous mixing below the cloud point to dissolve CRL1005, followed by warming above the cloud point to allow for particle formation. Samples were diluted before analysis as described in the Experimental section.

[b]Particle size of 0.2 and 2.5 mg/mL DNA, respectively, in PBS.

Adsorption of BAK to CRL1005 Particles

To determine the adsorption of BAK to CRL1005 particles, BAK at various concentrations was mixed either with cold (below cloud point) CRL1005 solution (7.5 mg/mL in PBS) or with a CRL1005 suspension at room temperature. After a 1-h incubation at room temperature, 1 mL of the mixture was centrifuged at 25°C for 1 h at 440,000*g*. The BAK concentration in the supernatant was determined using UV absorption and second-derivative analysis in the range of 230–300 nm using methodology developed previously for the analysis of protein and DNA mixtures.[14] A Hewlett-Packard 8453 diode-array spectrophotometer controlled by a personal computer equipped with the HP spectral analysis software was used. Solutions of BAK-12 and plasmid DNA in PBS were used as the reference standards for the analysis. The amount of adsorbed BAK was obtained by subtracting the BAK concentration in the supernatant from the total BAK concentration in the mixture.

BAK Composition Analysis

The homolog composition of BAK (Spectrum) was determined by reverse-phase HPLC (RP-HPLC) using an isocratic separation method on a YMC-Pack CN (cyano) 120A S-5 4.6 × 250 mm column at room temperature (Waters, Milford, MA). The mobile phase contained 55% acetonitrile and 45% 0.1 M sodium acetate at pH 5.0. BAK samples were injected at a flow rate of 1 mL/min and the UV absorbance was monitored at 260 nm. The chromatograms were transferred to an Excel spreadsheet for quantitative analysis. UV absorbance peaks were identified by comparison to BAK-12, BAK-14, and BAK-16 reference standards.

DNA Association to CRL1005-BAK Particles

The adsorption of DNA to CRL1005 particles was determined using a filtration method and/or by sucrose density gradient centrifugation. For the filtration method, 3- to 5-mL samples were filtered through 25-mm syringe filters with a pore size of 0.1 μm (MILLEX-VV; Millipore). The DNA concentration in the filtrate (free DNA concentration) was determined by the UV absorbance at 260 nm. The amount of DNA bound to CRL1005-BAK particles was calculated by subtracting the

free DNA from total DNA. Filtration of control samples containing only DNA in PBS indicated no detectable DNA adsorption to the filter.

A sucrose density gradient centrifugation method was also used to determine the association of DNA to CRL1005 particles. For BAK-containing samples, a 3.2-mL gradient containing 2–13% sucrose and 25 mM NaCl was used. Samples (0.2 mL) were layered on top of the gradient and centrifuged at 80,000 rpm (346,000*g*) for 1.5 h to pellet the free DNA. After centrifugation, 16 gradient fractions (0.2 mL) were collected and analyzed for DNA concentration by first dissolving the CRL1005 polymer with sodium dodecyl sulfate (to 1% w/w), then using a UV multicomponent analysis method to determine the DNA concentration.[14] Control studies conducted with samples containing CRL1005, DNA, DNA/CRL1005, and DNA/CRL1005/BAK in PBS indicated that the CRL1005 polymer remained in the upper 6–8 fractions of the gradient after centrifugation but that essentially all (>99%) of the free DNA was pelleted (data not shown). For samples containing CPC, CTAC, or high concentrations (>1.2 mM) of BAK, a 12–18% sucrose gradient in 25 mM NaCl was utilized. Higher levels of DNA association in these samples increased the density of the DNA-CRL1005-surfactant particles such that they would pellet through the 2–13% sucrose gradient. Centrifugation of the 12–18% gradient was performed at 346,000*g* for 4 h.

Microscopy

The morphology of the particles in CRL1005 formulations and DNA association to the particles were imaged using an Olympus IX71 microscope equipped with a Spot digital camera. Three microliters of the formulation was placed on a slide, covered with a glass coverslip, and sealed with nail polish. A polarizer condenser and a 100-power objective were used for bright field imaging of the particles to determine morphology. To visualize DNA association, large particles of CRL1005 (~2 μm) were formed by slowly warming a cold solution of 7.5 mg/mL CRL1005 in PBS above the cloud point. BAK, PicoGreen, and plasmid DNA were then added, in that order. The DNA concentration was lowered to 0.3 mg/mL to reduce the background fluorescence, and the BAK and PicoGreen concentrations were 0.6 mM and 1:1000 (v/v), respectively. The excitation/emission cube for fluorescein isothiocyanate was used for DNA/PicoGreen fluorescence.

Assay for DNA-BAK Precipitates in DNA/CRL1005/BAK Formulations

DNA-BAK precipitates were separated from free DNA by centrifugation as described below. One milliliter of each formulation was centrifuged (at 25°C) in a Beckman Ultracentrifuge with a fixed-angle rotor for 30 min at 5, 10, 15, 20, 25, and 35 thousand rpm (1.4 k, 5.4 k, 12.3 k, 21.8 k, 34 k, and 66.5 k*g*, respectively). DNA-BAK precipitates were completely pelleted at 20,000 rpm; however, free DNA did not start to pellet until the speed reached 35,000 rpm (~10% of the free DNA was pelleted at 35,000 rpm). There was no significant sedimentation of CRL1005-BAK-DNA particles at or below 35,000 rpm (data not shown). Pelleted precipitates of DNA-BAK were dissolved in 2% sodium dodecyl sulfate before UV spectroscopy to determine the DNA concentration.

RESULTS

Immunogenicity of CRL1005 Formulations in Rhesus Macaques

To evaluate the ability of CRL1005 and CRL1005-BAK to adjuvant the immune response induced by plasmid DNA, the immunogenicity of adjuvanted and unadjuvanted formulations of V1Jns-gag plasmid DNA in rhesus monkeys were compared. The results in Table 2 show that the addition of 7.5 mg/mL CRL1005 to the control formulation containing only 5 mg/mL V1Jns-gag DNA in PBS enhanced the gag-specific cellular immune response, based on an increase in IFN-γ-secreting T cells after the second immunization (at 6 weeks). However, the strongest immune responses were generated by a formulation containing 5 mg/mL DNA formulated with 7.5 mg/mL CRL1005 and 0.5 mM BAK. These results clearly show that BAK enhanced the immunogenicity of V1Jns-gag plasmid DNA.

Binding of BAK to CRL1005 Particles

To determine the effects of BAK on the size and morphology of CRL1005 particles, we formulated 5 mg/mL DNA with 7.5 mg/mL CRL1005 (formulation D113) and evaluated the effect of adding 0.6 mM BAK (to make formulation D118), using visual microscopy. As shown in Figure 1A, the CRL1005 particles in the absence of BAK were found to be quite uniform in size, with the

Table 2. Effect of CRL1005 and BAK on the Immune Response Induced by HIV *gag* Plasmid DNA in Rhesus Monkeys[a]

	Gag-Specific IFN-γ ELISPOT Response (SFC per 10^6 PBMCs) at Week:				
Vaccine Formulation	0	4	6	8	12
5 mg/mL *gag* DNA	0	5	33	28	139
	1	9	81	9	7
	3	10	82	26	96
	1	30	82	45	71
	1	**14**	**70**	**27**	**78**
5 mg/mL *gag* DNA	18	19	116	61	182
7.5 mg/mL CRL1005	4	39	168	125	283
	0	13	219	98	122
	4	7	125	57	216
	7	**20**	**157**	**85**	**201**
5 mg/mL *gag* DNA	0	108	530	366	380
7.5 mg/mL CRL1005	7	486	671	374	1405
0.5 mM BAK	2	5	111	31	53
	0	54	152	77	161
	2	**163**	**366**	**212**	**500**

[a]Plasmid DNA (5 mg) was injected im into four rhesus monkeys per group at 0, 4, and 8 weeks. At the indicated times, the gag-specific T cells were quantified using the IFN-γ ELISPOT assay as described in the Experimental section. Results shown are net responses after subtraction of spots formed in medium control wells. The mean response for each group is shown in bold typeface for each time point. SFC = spot forming cells.

majority of the particles being <5 microns in diameter. However, the addition of BAK reduced the apparent particle diameter to significantly below 2 microns. To confirm the effect of BAK on the particle size, we used static laser diffraction light scattering on the same plasmid DNA-containing formulations. The results (Fig. 1B) show that the mean CRL1005 particle size in the absence of BAK (D113) was ~2–3 microns, whereas in the formulation containing BAK (D118), the CRL1005 particles had a diameter of ~300 nm. We also prepared a formulation containing 5 mg/mL DNA and 0.6 mM BAK in PBS (without CRL1005) to determine the potential for generating DNA-BAK precipitates in D118. The results indicate that DNA-BAK particles were present (without CRL1005) and, based on the particle-size distribution shown in Figure 1B, most of volume of the particles was associated with particles having a diameter in the 100- to 1000-micron range. The cloudy visual appearance of this formulation also confirmed the presence of large particles.

To examine the binding of BAK to CRL1005 particles, BAK was mixed with 7.5 mg/mL CRL1005 in PBS either before or after particle formation and the amount of BAK bound to the CRL1005 particles was determined as described in Experimental. The results in Figure 2 show that ~50% of the input BAK was bound to CRL1005 particles and that the amount of BAK bound was the same regardless of whether the BAK was present during particle formation or was added after particle formation. The results also indicate that the percentage of BAK bound to CRL1005 particles was slightly higher in samples prepared with BAK from Ruger Chemical, compared with samples containing BAK from Spectrum, suggesting that the BAK composition affects binding to CRL1005 particles (discussed below).

Light-scattering measurements indicated that the hydrodynamic diameter of CRL1005 particles formed in the presence of BAK was ~300 nm (Fig. 1B). However, when CRL1005 particles were formed in the absence of BAK then mixed with BAK, the particle size before and after BAK addition was the same (2.6 microns). Therefore, the results in Figure 2 show that the binding of BAK to CRL1005 particles is independent of surface area, because the particle size (and surface area) did not affect the amount of BAK bound to the particles. The data suggest that BAK not only binds to the surface of the particles but also penetrates into (or is soluble in) the interior of the CRL1005 particle. Measurements of the partition coefficient of BAK, between a pure CRL1005 polymer phase and an

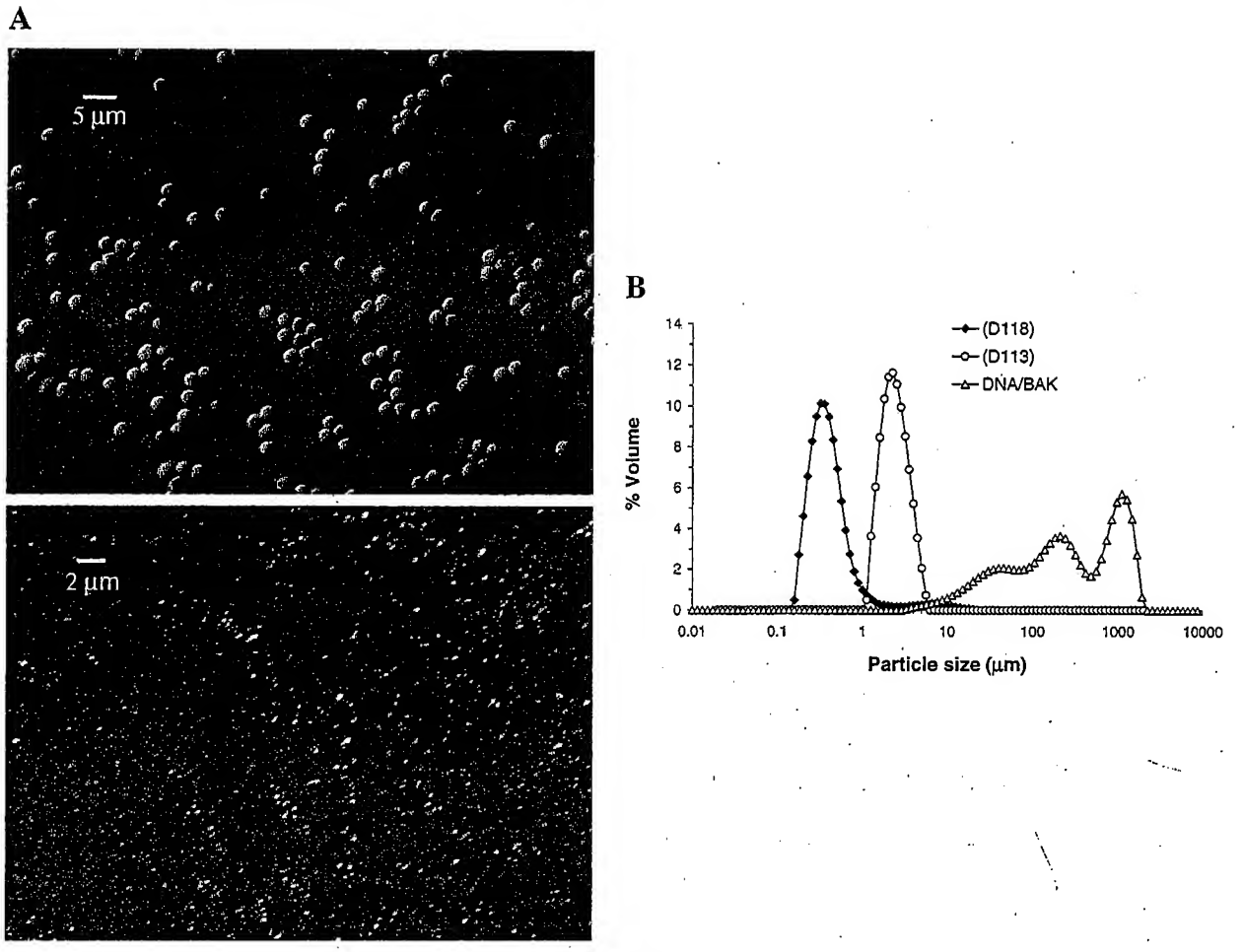

Figure 1. Morphology and size distribution of CRL1005 particles. (A) The morphology of CRL1005 particles was visualized by microscopy as described in the Experimental section. In a PBS formulation containing 5 mg/mL plasmid DNA and 7.5 mg/mL CRL1005 but no BAK (D113), the CRL1005 particles had a smooth and spherical morphology with a diameter of ~2 μm (upper panel). In a PBS formulation containing 5 mg/mL plasmid DNA, 7.5 mg/mL CRL1005 and 0.6 mM BAK (D118), the CRL1005 particle size was reduced to <0.5 μm (lower panel). (B) Particle-size distribution determined by static laser diffraction light scattering. The DNA/BAK mixture contained 5 mg/mL DNA and 0.6 mM BAK. Each formulation was warmed from −70°C storage to room temperature, then diluted with PBS before the particle-size measurement.

aqueous phase containing PBS, strongly suggest that BAK preferentially partitions into the CRL1005 polymer. The partition coefficients for BAK-12 and BAK-14 into the CRL1005 phase are 59 and 380, respectively.

Effect of BAK Chain Length on Binding to CRL1005 Particles

The results in Figure 2 indicate that BAK binds to CRL1005 particles. However, commercially available BAK is a mixture of several homologs with the hydrocarbon chain having lengths of 12, 14, 16, and sometimes 18 carbons. Because hydrophobic interactions are the likely driving force for the binding of BAK to CRL1005 particles, we determined the effect of BAK chain length on its ability to associate with particles of CRL1005. Our approach relied on the use of RP-HPLC to analyze the BAK in the unbound fraction, after centrifugation to pellet CRL1005 particles with bound BAK. The results shown in Table 3 indicate that >95% of the unbound BAK fraction was BAK-12, showing that the longer chain-length

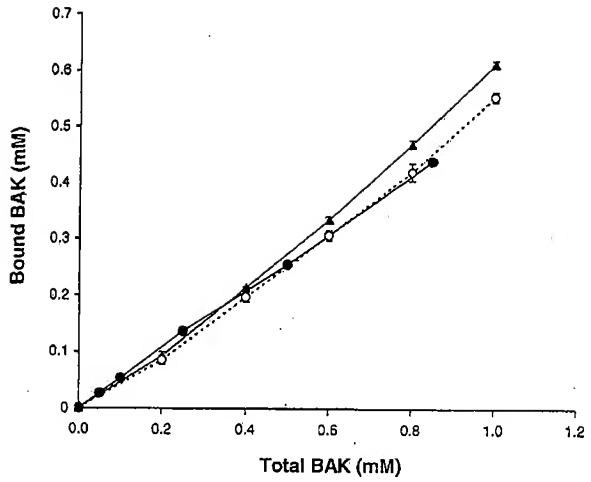

Figure 2. Adsorption of BAK to CRL1005 particles. In solutions containing CRL1005 and varying concentrations of BAK (without DNA), the CRL1005 particles with adsorbed BAK were pelleted by centrifugation, and the free BAK concentration in the supernatant was determined by UV absorption and second-derivative analysis, as described in the Experimental section. BAK at the designated concentrations and CRL1005 at 7.5 mg/mL were mixed using two different methods: (1) The CRL1005/BAK mixture was chilled on ice to obtain a clear solution, then warmed to room temperature, allowing CRL1005 particles to form in the presence of BAK (from Spectrum). The CRL1005 particles formed under these conditions had a diameter of ~300 nm (○); (2) CRL1005 in PBS at 7.5 mg/mL was chilled on ice to obtain a clear solution, then warmed to room temperature to allow the CRL1005 polymer to form ~2-μm particles in the absence of BAK. After particle formation, 0.6 mM BAK (from Spectrum) was added with gentle mixing (●); (3) same as (2) except BAK was from Ruger Chemical (▲).

homologs have a greater tendency to associate with CRL1005 particles. Comparing these results with those in Figure 2 indicates that the percentage of BAK bound to the CRL1005 particles was ~25%, ~90–95%, and ~100% for BAK-12, BAK-14, and BAK-16, respectively, in the presence and absence of 5 mg/mL plasmid DNA. These results strongly suggest that hydrophobic interactions are the major driving force for association of BAK to CRL1005 particles. The composition of BAK from Ruger Chemical (50% BAK-14-18, composition provided by the vendor) has a somewhat larger fraction of the longer chain-length homologs than Spectrum BAK (~33% BAK-12-16), as measured by RP-HPLC. Based on the results in Table 3 and the known compositions of Spectrum and Ruger BAK, we would predict that a larger fraction of the total BAK would be bound to CRL1005 particles for Ruger BAK. The results in Figure 2 are consistent with this prediction.

Table 3. Composition of Unbound BAK in CRL1005 Formulations

	Percent of each BAK Homolog		
Sample	BAK-12	BAK-14	BAK-16
Spectrum BAK control	67.4	24.2	8.3
Supernatant A[a]	97.9	1.9	0.2
Supernatant B[b]	96.7	2.7	0.7

[a]Supernatant from a sample containing 7.5 mg/mL CRL1005 and 0.85 mM Spectrum BAK.
[b]Supernatant from a sample containing 7.5 mg/mL CRL1005, 0.85 mM Spectrum BAK, and 5 mg/mL DNA.

Effect of BAK on the Size and Surface Charge of CRL1005 Particles

The effect of BAK on the zeta potential and size of CRL1005 particles is shown in Table 1. The results show that the CRL1005 particles had a hydrodynamic diameter of ~2 microns and a surface charge close to neutrality, as expected from the nonionic structure of the polymer. The addition of 5 mg/mL DNA to the CRL1005 preparation resulted in only a minor change in the zeta potential and particle size. Moreover, the results with the DNA control show that the plasmid DNA in the CRL1005 formulations did not dominate the zeta potential or the particle-size measurements. The zeta potential of a formulation containing 0.6 mM BAK and 7.5 mg/mL CRL1005 was ~+10 mV, a value consistent with the data in Figure 2 showing that BAK binds to CRL1005 particles. The presence of BAK during particle formation also caused a large reduction in particle size. The addition of plasmid DNA to the CRL1005/BAK formulation produced particles with a zeta potential of ~−47 mV and a diameter near 330 nm. The highly negative zeta potential suggests that DNA binds to CRL1005-BAK particles, giving them a negative surface charge.

The effect of BAK and CRL1005 concentration on the zeta potential of CRL1005 particles in the presence of 5 mg/mL DNA is shown in Figure 3. At 7.5 mg/mL CRL1005, the results show that CRL1005 particles became negatively charged at ≥0.4 mM BAK but that there was no detectable surface charge at or below 0.3 mM BAK. These results suggested that a threshold of positive surface charge is required for binding of DNA to CRL1005-BAK particles. To further explore the

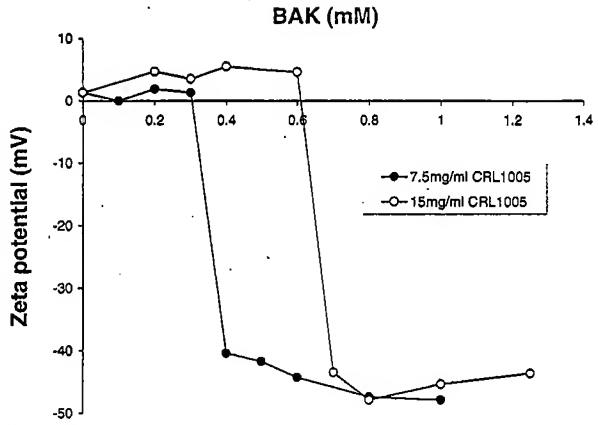

Figure 3. Effect of BAK and CRL1005 concentration on the zeta potential of CRL1005 particles. Formulations containing 7.5 or 15 mg/mL CRL1005, 5 mg/mL plasmid DNA in PBS, and varying concentrations of BAK were diluted and the zeta potential measurements made as described in the Experimental section.

nature of this threshold, we repeated the experiment using a twofold-higher concentration of CRL1005. We reasoned that if the threshold corresponds to the minimum positive surface charge required for DNA binding, then doubling the CRL1005 concentration would result in a doubling of the BAK concentration at the threshold. The results in Figure 3 are consistent with this hypothesis. However, the data in Figure 2 indicate that there was no threshold of BAK concentration required for BAK binding to CRL1005 particles. Therefore, the threshold observed in Figure 3 suggests that a minimum positive surface-charge density is required for the binding of DNA to CRL1005-BAK particles.

DNA Binding to CRL1005-BAK Particles

To further determine whether plasmid DNA binds to CRL1005 particles in the presence or absence of BAK, we subjected samples containing 7.5 mg/mL CRL1005, 5 mg/mL DNA, and various concentrations of BAK to sucrose gradient centrifugation analysis to separate unbound DNA from the CRL1005 polymer. UV spectroscopic analysis of fractions of the sucrose gradient after centrifugation revealed that there was no detectable DNA in the polymer containing fractions in the absence of BAK (Fig. 4A). However, there was a significant amount of DNA in the polymer containing fractions in the presence of BAK, suggesting that DNA binds to CRL1005 particles only in

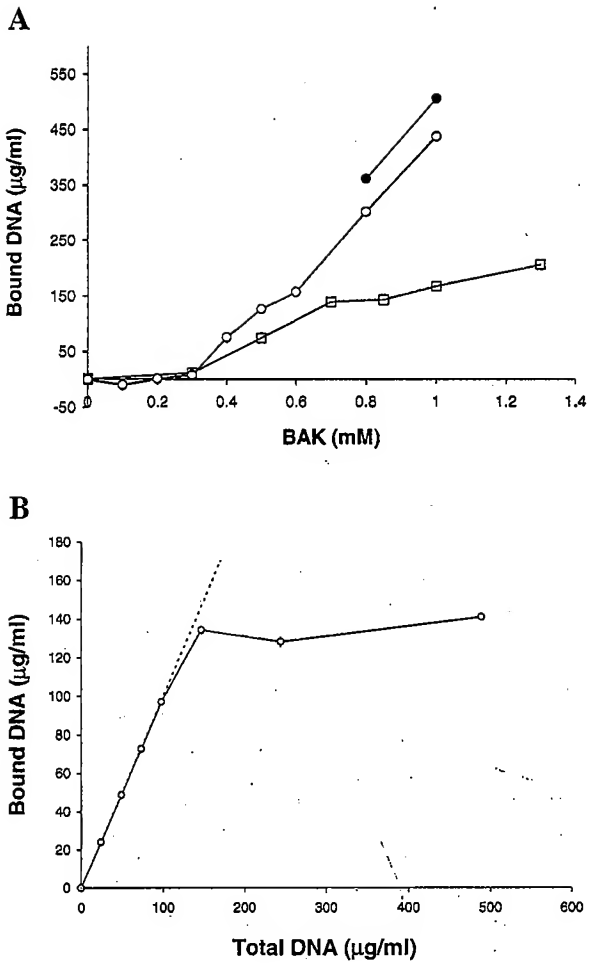

Figure 4. (A) Effect of BAK concentration on the amount of plasmid DNA associated with CRL1005 particles. Samples contained 7.5 mg/mL CRL1005, 5 mg/mL plasmid DNA, and the indicated concentrations of BAK. Free DNA was separated from DNA bound to CRL1005 particles using a sucrose density gradient centrifugation method (□) or a filtration method with 0.5 mg/mL DNA (○) and 5 mg/mL DNA (●). The amount of DNA bound to CRL1005 particles is expressed as micrograms/milliliter bound DNA with a total DNA concentration of 5 mg/mL. (B) Effect of DNA concentration on the amount of DNA bound to CRL1005 particles. DNA association to particles in a formulation containing 7.5 mg/mL CRL1005, 0.6 mM BAK, and the indicated concentration of DNA, was quantitated by filtration and UV spectroscopy, as described in the Experimental section. The dashed line represents expected value, assuming 100% binding of plasmid DNA.

the presence of BAK. The results also show that the amount of DNA associated with the CRL1005 particles increased with BAK concentration and that the amount of DNA bound at 0.5 mM BAK

(~75 μg/mL) represented about 1.5% of the total DNA. Because the amount of DNA bound to the polymer particles was such a small fraction of the total DNA, a second approach was used to confirm the presence of bound DNA, based on a filtration method to separate CRL1005 particles from free DNA. The results (Fig. 4A) show that DNA was retained by the filter only in the presence of BAK, suggesting that there was no significant binding of DNA to CRL1005 particles in the absence of BAK. However, the amount of DNA retained with the CRL1005-BAK particles was significantly higher using this technique than with the sucrose gradient centrifugation method (above 0.6 mM BAK). Because the background level of DNA retained by the filter in the absence of CRL1005 and BAK was affected by the DNA concentration, this titration was conducted at 0.5 mg/mL DNA. However, repeating the analysis at 0.8 and 1.0 mM BAK using the filtration method at 5 mg/mL DNA provided similar results. These results suggest that plasmid DNA binds to CRL1005 particles in the presence of BAK and that the amount of DNA binding at 0.6 mM BAK is ~100–150 μg/mL, which represents ~2–3% of the total DNA. It is not entirely clear why the results for the centrifugation and filtration methods differ significantly above 0.6 mM BAK but it seems likely that some of the bound DNA may be lost from the DNA-CRL1005-BAK particles during the centrifugation method because the sample gets diluted by ~16-fold during centrifugation. We do not believe that the presence of DNA-BAK precipitates was responsible for these differences, because there is no evidence for their existence above the cloud point of the formulation (Fig. 7, discussed below).

To determine how DNA binding to CRL1005 particles is affected by the DNA concentration, a sample containing 0.6 mM BAK and 7.5 mg/mL CRL1005 was titrated with increasing amounts of DNA and the amount of DNA bound to the CRL1005 particles was determined by filtration. The results shown in Figure 4B indicate that the amount of DNA bound to the CRL1005 particles saturated at ~135 μg/mL DNA. Because ~100% of the DNA was bound to CRL1005 particles at concentrations ≤100 μg/mL, these data suggest that DNA binds to CRL1005-BAK particles with relatively high affinity. The maximum capacity for binding of DNA in this formulation (~135 μg/mL) corresponds to ~2.7% of the total DNA in a formulation containing 5 mg/mL plasmid DNA.

Microscopic Visualization of Plasmid DNA Bound to CRL1005-BAK Particles

To determine whether DNA bound to CRL1005 particles could be visualized by light microscopy, we first formed CRL1005 particles of ~2-μm diameter by warming a cold solution of CRL1005 (7.5 mg/mL) in PBS to room temperature. BAK (0.6 mM) was then added to allow for binding to the preformed CRL1005 particles. After addition of the DNA-specific fluorescent label PicoGreen (Molecular Probes) and 0.3 mg/mL plasmid DNA, the particles were observed using a fluorescent microscope. The images shown in Figure 5 clearly

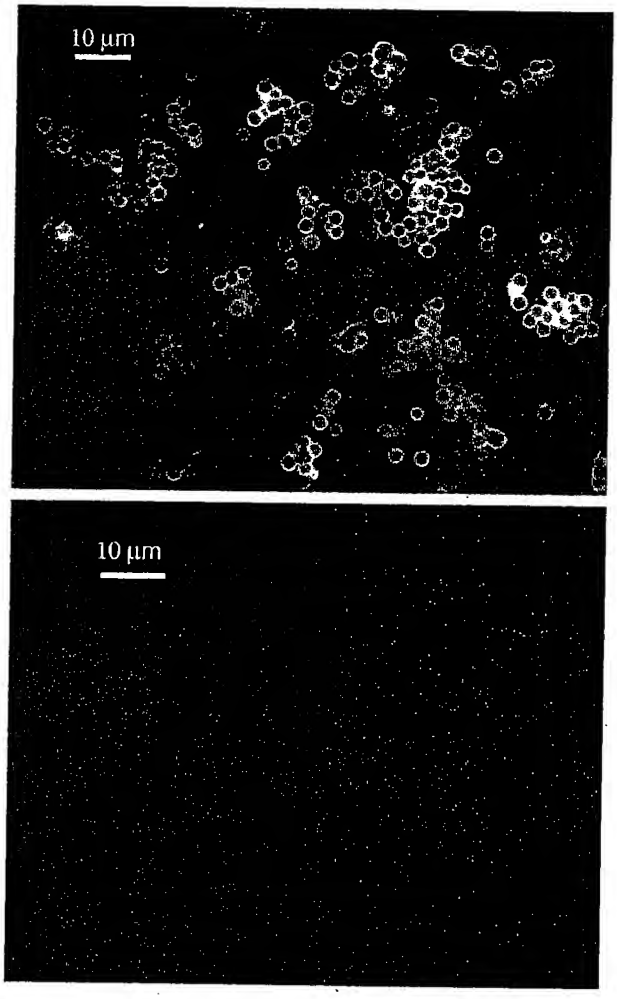

Figure 5. Fluorescence microscopy of CRL1005 particles in the presence of DNA. CRL1005 particles were prepared in the absence of BAK (~2-μm diameter). After particle formation, BAK, the DNA fluorescent probe PicoGreen, and DNA were added in order (upper panel). A DNA concentration of 0.3 mg/mL was used to reduce background fluorescence. As a control, a sample was prepared by the above procedure, but without BAK (lower panel).

show fluorescent rings of labeled DNA of the expected size. The results show no evidence of DNA binding to CRL1005 particles in the absence of BAK.

Although the composition of the formulation prepared for the visualization of DNA binding to CRL1005-BAK particles was not exactly the same as the formulation used in the rhesus monkey studies (D118), the same CRL1005 concentration (7.5 mg/mL) and buffer (PBS) were used. Because the particles in the D118 formulation were too small for effective visualization (~300 nm), it was necessary to generate ~2-micron particles by slowly warming the CRL1005 solution through the phase-transition temperature. However, the results in Figure 2 indicate that the CRL1005 particle size does not affect the binding of BAK; therefore, the amount of BAK bound to the CRL1005 particles in this formulation was the same as in D118. Because the use of 5 mg/mL plasmid DNA produced excessively high background fluorescence, the DNA concentration was reduced to 0.3 mg/mL. However, this change would not be expected to reduce the amount of DNA bound to the CRL1005-BAK particles, because the results shown in Figure 4B show that the binding of plasmid DNA to CRL1005-BAK particles saturates at ~150 μg/mL plasmid DNA. Therefore, these results strongly suggest that plasmid DNA binds to the surface of CRL1005-BAK particles in the same formulation used to generate the immunogenicity results in rhesus monkeys (D118).

Effect of Other Cationic Surfactants on the Binding of DNA to CRL1005 Particles

To determine whether other cationic surfactants would enhance the binding of plasmid DNA to CRL1005 particles, we titrated samples of 7.5 mg/mL CRL1005 with increasing concentrations of CPC and CTAC, then separated bound from free DNA. The results shown in Figure 6 show that each of these surfactants was able to support the binding of DNA to CRL1005 particles at concentrations above 0.2 mM, in a concentration-dependent manner. The results also show that the level of DNA binding was similar for each surfactant and similar to the results with BAK, shown in Figure 4A. Interestingly, these data also suggest the existence of a threshold of surfactant concentration required for the binding of DNA, but the threshold appears to be at ~0.1–0.2 mM CPC/CTAC. In a separate study, we titrated a formulation containing 22.5 mg/mL CRL1005 and 5 mg/mL DNA with increasing concentrations of CPC and CTAC and the results clearly showed a threshold at 0.5 mM for both surfactants (data not shown). If our hypothesis for the existence of this threshold is correct, we would expect the threshold to be approximately threefold higher at 22.5 mg/mL than at 7.5 mg/mL and this would correspond to a threshold of ~0.17 mM surfactant for CPC and CTAC with 7.5 mg/mL CRL1005 (a result that is consistent with the data shown in Fig. 6).

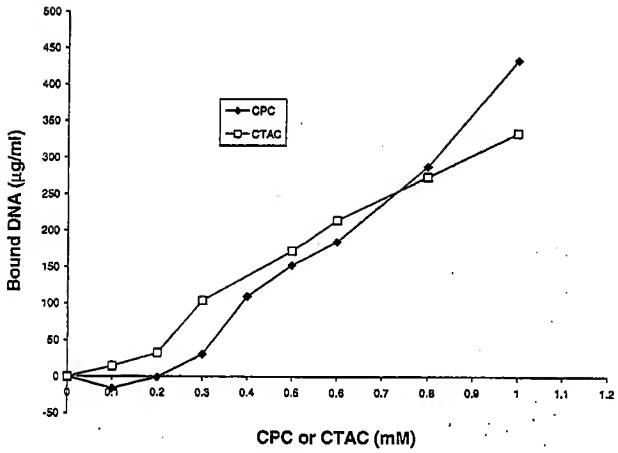

Figure 6. Binding of DNA to CRL1005 particles in the presence of CPC or CTAC. Samples contained 5 mg/mL plasmid DNA, 7.5 mg/mL CRL1005, and one of the surfactants at the indicated concentrations. Free DNA was separated from DNA bound to CRL1005 particles using the filtration method. The amount of DNA bound to CRL1005 particles is expressed as micrograms/milliliter in a sample containing 5 mg/mL plasmid DNA.

Dissolution of DNA-BAK Precipitates by CRL1005

It has been reported that DNA-BAK precipitates are able to enhance the immune response induced by plasmid DNA in mice.[15] Based on this report, it seemed possible that the immune response induced by DNA/CRL1005/BAK formulations (see Fig. 1) might have been due to the presence of DNA-BAK precipitates. To determine whether DNA-BAK precipitates are present in DNA/CRL1005/BAK formulations above the cloud point, PBS formulations containing DNA only, DNA-BAK, or DNA/CRL1005/BAK were subjected to a step centrifugation method to separate free DNA from DNA-BAK precipitates and from CRL1005-BAK-DNA particles. The results, shown in Figure 7, indicate that there was ~100 μg of residual DNA recovered from the bottom of the centrifuge tube in the absence of

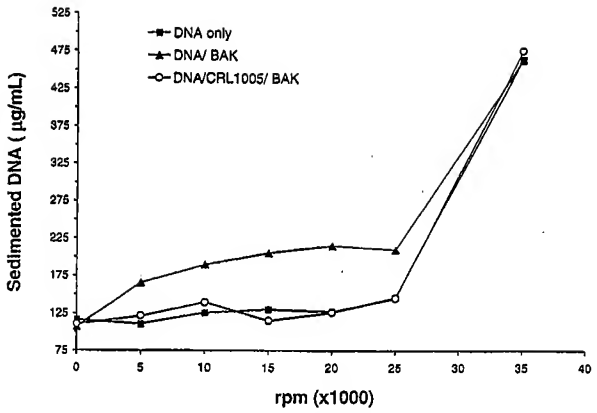

Figure 7. Complete dissolution of DNA/BAK precipitates above the cloud point of CRL1005. DNA sedimented during 30-min centrifugation steps at various speeds for solutions containing only plasmid DNA, or a solution containing 5 mg/mL DNA and 0.5 mM BAK, or a solution containing 5 mg/mL DNA + 7.5 mg/mL CRL1005 + 0.5 mM BAK. The amount of DNA sedimented is expressed as micrograms/milliliter in a sample containing 5 mg/mL plasmid DNA.

centrifugation for the DNA-only control; therefore, this level of DNA was considered background. The amount of DNA pelleted in the DNA-only control did not substantially increase above the background level until the centrifugation speed exceeded 25,000 rpm; therefore, no significant amount of free DNA was pelleted at or below 25,000 rpm. Moreover, there was no significant difference in the amount of DNA sedimented at ≤25,000 rpm between the DNA control and the DNA/CRL1005/BAK formulation, suggesting that DNA-BAK precipitates were not present (CRL1005-BAK-DNA particles did not sediment until >35,000 rpm). However, in formulations containing DNA and BAK (without CRL1005), the amount of sedimented DNA was significantly greater than that of the DNA control or the DNA/CRL1005/BAK formulation (~100 µg/mL over background at 20,000 rpm). Therefore, these data indicate that there was no detectable amount of DNA-BAK precipitate present in the DNA/CRL1005/BAK formulation above the cloud point. Because visual observation of this formulation below the cloud point (data not shown) clearly reveals the presence of DNA-BAK precipitates (in contrast, CRL1005/DNA formulations are completely clear below the cloud point), these results suggest that CRL1005 particles completely dissolve any DNA-BAK particulates. This conclusion is also supported by the light-scattering measurements of D118, shown in Figure 1B, which show no indication of the presence of DNA-BAK precipitates above the cloud point. Additional light-scattering measurements of D118-like formulations containing even higher concentrations of BAK (up to 1.0 mM) also showed no evidence for the presence of DNA-BAK precipitates (data not shown). Taken together, these data indicate that the enhancement of cellular immunity induced by the D118 formulation was not due to DNA-BAK precipitates.

DISCUSSION

Based on the need to identify safe and effective adjuvants for plasmid DNA vaccines, we began this work by exploring the adjuvant properties of CRL1005. The initial animal studies showed that CRL1005 enhances the cellular immune response induced by HIV-1 *gag* plasmid DNA in rhesus monkeys. Because the adjuvant properties of CRL1005 are believed to be related to its ability to form particles,[6,7] we conducted biophysical characterization studies in an attempt to correlate the physical/chemical properties of the formulation with the enhanced immune response. These initial studies included measurements of associated DNA. We thought it unlikely that the CRL1005 particles would bind plasmid DNA, because it is a nonionic polymer, and our results confirmed this hypothesis. However, we believed that a hydrophobic cationic surfactant would be likely to bind to the surface of the CRL1005 particles rendering them capable of binding DNA through electrostatic interactions. Based on previous reports of enhanced DNA delivery by cationic microparticles with DNA adsorbed to the surface,[16,17] we hypothesized that cationic microparticles of CRL1005, produced by formulating CRL1005 with a cationic surfactant, might also enhance DNA delivery. We selected BAK as the cationic surfactant to test this hypothesis, in rhesus immunogenicity studies, based on its history of use in pharmaceutical products.[18] Moreover, the BAK concentration was selected to be within the concentration range used in previous pharmaceuticals, to minimize the possibility of adverse responses. The DNA concentration was selected on the basis of previous immunogenicity results using nonadjuvanted DNA formulations.

The immunogenicity and characterization results demonstrate that the addition of BAK to a

DNA/CRL1005 formulation leads to the formation of CRL1005-BAK-DNA particles and to an enhancement of the cellular immune response in rhesus monkeys. The mechanism for how the CRL1005-based plasmid DNA formulations enhance the immune response is unknown; however, it seems possible that the DNA-CRL1005-BAK particles may be acting as an adjuvant and/or enhancing the delivery of plasmid DNA.

Aluminum phosphate is a negatively charged particle-based adjuvant that is capable of enhancing the immune response induced by DNA vaccines.[8,19–21] Although aluminum phosphate particles are much larger than the DNA-CRL1005-BAK particles, the particulate nature and negative surface charge of the DNA-CRL1005-BAK particles suggest that they may be functioning similarly to aluminum phosphate. However, there is also significant support for the hypothesis that cationic microparticles can enhance the delivery of DNA, *in vivo*. One such cationic microparticle formulation is based on the use of PLG (polylactide-*co*-glycolide) and CTAB (cetyltrimethylammonium bromide) to produce cationic microparticles that bind DNA.[16] The PLG-CTAB microparticles are reported to be more effective than naked DNA for induction of immune responses in mice, guinea pigs, and rhesus monkeys.[22] Moreover, the mechanism of action appears to be, at least in part, the facilitation of DNA uptake by antigen-presenting cells.[23] Another type of cationic microparticle formulation being evaluated as a gene-delivery vehicle was prepared by adding the cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) to an oil-in-water emulsion (MF59 and other emulsions) to make a cationic submicron emulsion with a particle size near 180 nm. Based on immunogenicity data generated in mice and rabbits, the MF59/DOTAP emulsion appears to be an effective delivery system for plasmid DNA.[17]

A significant difference between the previously described cationic microparticle formulations and CRL1005-based formulations containing BAK, is the polarity of the surface charge after DNA binding. For the two cationic microparticle formulations described above, the net surface charge after binding DNA is positive,[16,17] but CRL1005-BAK-DNA particles have a negative surface charge. The significance of surface-charge polarity on the type and/or magnitude of the immune responses induced by plasmid DNA is unknown, but the difference suggests that CRL1005-BAK-DNA particles may not have the same mechanism of action as the cationic microparticle formulations previously described. It should also be noted that Prokop et al.[24] evaluated a series of cationic polymers for their ability to enhance *in vivo* gene delivery and showed that the most effective polymers were members of the Tetronic polymer series (polyethylene–polypropylene block copolymers; BASF) that formed negatively charged particles with DNA. Therefore, the influence of surface-charge polarity on the ability of a microparticle carrier to deliver plasmid DNA may be different in different formulations or in different animal species.

One characteristic of the DNA/CRL1005/BAK formulation suggesting that increased DNA delivery is an unlikely explanation for the enhanced immune response is the low level of DNA associated to the CRL1005-BAK particles. In the D118 formulation containing 0.5–0.6 mM BAK, the amount of DNA associated to CRL1005-BAK particles is ~1.5–3% of the total DNA (at 5 mg/mL). Although the level of bound DNA is a small fraction of the total, it corresponds (by calculation) to ~20–40 molecules of plasmid bound to each of the $\sim 5 \times 10^{11}$ CRL1005 particles/mL (based on a monodisperse population of ~300-nm spherical particles and a 7-Kb plasmid). Based on the high concentration of DNA-CRL1005-BAK particles and the presence of multiple copies of plasmid on each particle, these calculations suggest that enhanced DNA delivery is a reasonable hypothesis to explain the increased immune response caused by the addition of BAK to the DNA/CRL1005 formulation. Unfortunately, it is not possible to address this hypothesis in animals by testing a formulation containing only ~150 μg/mL total DNA, to eliminate the immune response induced by free DNA, because the use of DNA concentrations in this range results in extensive aggregation. Moreover, experiments to examine the delivery of plasmid by measuring transgene expression in mice does not appear to be feasible because of saturation of DNA delivery at DNA concentrations far below the 5 mg/mL DNA concentration in the D118 formulation. These results indicate that studies to address the mechanism of immune enhancement will need to be done in animals larger than mice and with formulations having a DNA concentration ≥~1 mg/mL.

Our results indicate that BAK acts to stabilize small (~300 nm) CRL1005 particles and to bridge the association of DNA to the particle surface. Moreover, based on the data in Table 3 and Figure 2, the BAK-14 and BAK-16 homologs were

nearly completely bound to CRL1005 particles, but ~75–80% of the BAK-12 remained unassociated. The effects of free BAK-12 on the immunogenicity are unknown, but the mere presence of free surfactant (~0.25 mM BAK-12 in D118 with 0.5 mM total BAK) in the formulation raises the possibility that it may facilitate DNA delivery by enhancing the permeability of cell membranes or by acting through an unknown pathway. However, the surfactant properties of BAK may also have an inhibitory effect on antigen-presenting cells. The results in Table 3 suggest that a CRL1005/BAK/DNA formulation containing only BAK-14 and/or BAK-16 (or CPC or CTAC) would have greatly reduced levels of free surfactant as well as a lower total surfactant concentration. Therefore, the effect of BAK on the immune response could be evaluated by conducting immunogenicity experiments comparing formulations prepared with the BAK mixture with those containing only BAK-14 or BAK-16.

Plasmid DNA and BAK surfactants form precipitates that have been reported to enhance DNA delivery.[15] Based on this report, an unexpected result from our studies is that DNA-BAK precipitates do not coexist with CRL1005-BAK-DNA particles (ternary complexes) in these formulations above the cloud point of CRL1005. However, DNA-BAK precipitates were observed in the D118 formulation below the cloud point (in the absence of CRL1005 particles). Further investigations using other biophysical techniques revealed a critical BAK concentration threshold required for interaction with DNA and that the free BAK concentration in the D118 formulation (above the cloud point) is below this threshold (results prepared for a separate publication). Taken together, these results suggest that BAK binds with higher affinity to CRL1005 particles than to plasmid DNA and that the presence of CRL1005 particles reduces the free BAK concentration below the threshold for interacting with DNA.

The DNA/CRL1005/BAK formulations described in this report appear to have significant advantages over other types of microparticle formulations in terms of the ease of manufacture and use, flexibility, and storage stability. They are liquid formulations, rather than lyophilized, and are therefore less costly to manufacture and easier to use because they do not require a separate diluent and a reconstitution step. Preparation of sterile vaccine formulations requires only the addition of sterile BAK to a solution of DNA/CRL1005 that is sterile-filtered below the cloud point. Moreover, the particle formation process for CRL1005 appears to be very reproducible. The size of CRL1005-BAK-DNA particles in the D118 formulation was consistently in the 250- to 350-nm range, even after repeated freeze/thaw/warming cycles, and was not significantly affected by the warming rate through the cloud point (data not shown).

In terms of flexibility, we have found that the formulation composition, surfactant type and concentration, and the CRL1005 concentration, can be adjusted to produce particle sizes from ~150 nm to 2–3 microns and DNA loading levels from 0 to >10,000 copies of plasmid per CRL1005 particle (unpublished data). Although CRL1005 particles appear to bind surfactants through hydrophobic interactions, the zeta potential measurements indicate that a fraction of the hydrophilic head groups are located on the surface of the particle and can bind to DNA molecules in solution. These data suggest that systematically altering the chemical structure of the surfactant headgroup might provide a convenient approach for exploring the effects of surface chemistry on the ability of CRL1005 particles to act as an adjuvant or a carrier for plasmid DNA vaccines.

Safety of the excipients used in a parenteral formulation is an important factor affecting regulatory approval and the acceptability of a vaccine. Therefore, it is highly desirable to use excipients already approved by regulatory agencies for new vaccine formulations. With regard to DNA/CRL1005/BAK formulations, BAK is approved by the FDA for use as a preservative.[25] Although it is most often used in ophthalmic products,[18] it is also approved for im injection.[25] CRL1005 has been evaluated in a Phase I clinical trial at doses up to 75 mg im without evidence of significant local or systemic toxicity.[26] Moreover, DNA/CRL1005/BAK formulations were well tolerated by both mice and rhesus monkeys (data not shown). These data suggest that the DNA/CRL1005/BAK formulation (D118) would be reasonable to test for use in humans.

The usefulness of a vaccine formulation can be limited by insufficient storage stability. Therefore, one of the goals of our laboratory is the development of DNA/CRL1005/BAK formulations that are stable for at least 2 years, when stored at 2–8°C. Compared with most protein-based or live-virus-based vaccines, plasmid DNA is very stable. In fact, we have previously reported the identification of plasmid DNA formulations (without BAK or CRL1005) that are stable for at least 2 years when

stored at room temperature.[27] However, the effects of BAK and CRL1005 on the stability of plasmid DNA have not been reported. In addition to any effect of CRL1005 and BAK on the stability of DNA, the stability of the CRL1005 polymer and BAK must also be ensured. BAK is known to be stable through autoclaving and is therefore unlikely to be a cause for concern.[18] However, POE-POP-POP polymers are known to degrade by autoxidation.[28] Therefore, the development of stable DNA/CRL1005/BAK formulations may require control of CRL1005 degradation. By monitoring both the physical and chemical stability of candidate formulations during storage, we have identified and characterized liquid formulations of DNA/CRL1005/BAK that are stable for at least 1 year at 2–8°C, suggesting that development of a stable liquid formulation is possible (results to be reported separately).

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